The pulmonate mud snail, *Amphibola crenata* as a potential bioindicator of estuarine trace metal and nutrient pollution in New Zealand

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ABSTRACT

The present study was the first to examine the toxicological, biochemical, physiological, and population level responses of an estuarine pulmonate snail, *Amphibola crenata,* to trace metal and nutrient pollution using both laboratory and field-based studies. The main objectives of this study were to (a) determine toxic mechanisms of *A. crenata* to both acute and chronic cadmium (Cd) exposure, and to investigate bioaccumulation of Cd in different tissues as a function of exposure concentrations and time; (b) assess multiple biomarker responses of field-collected snails in relation to trace metal and nutrient pollution levels present; and (c) evaluate the potential utility of *A. crenata* as a bioindicator for estuarine contamination in NZ.

The laboratory-based acute toxicity test showed that *Amphibola crenata* is highly tolerant to waterborne Cd, at least over acute (48 h) exposures. This high tolerance to Cd could be associated with large internal energy reservoirs (e.g. glycogen reserves), efficient antioxidant defences (e.g. catalase activity) and mechanisms that possibly restrict Cd exposure (e.g. mucus production and/or opercular closure). Exposure to chronic (21 days) Cd has shown more detrimental effects on mud snail health. Long-term exposure to Cd significantly induced oxidative stress in *A. crenata,* represented by tissue catalase activity and lipid peroxidation. Under both acute and chronic exposures, Cd induced a cascade of toxicological, physiological, and biochemical effects in *A. crenata*. During both exposures, individuals of *A. crenata* showed a significant accumulation of Cd in their tissues relation to availability of Cd in the seawater, demonstrating that this mud snail is a net accumulator of metal contaminants from their surroundings. The acute toxicity responses, however, indicated that short-term exposure to high levels of Cd may result in saturation of Cd in certain tissues including viscera and remaining tissues (i.e., kidney, mantle, remaining digestive tissues and heart) with increasing Cd concentration. During both acute and chronic exposure to Cd, oxygen consumption of mud snails was significantly reduced, with a concomitant increase in excretion rate. In addition, significant declines in O:N ratio and tissue glycogen levels were observed in Cd-exposed mud snails. The reduced O:N ratio suggests that protein is being mobilized for use as an energy source in this species, while depletion of tissue glycogen could be attributed to the utilisation of glycogen reserves by the organism through glycogenesis, to meet energy demand during stress. Catalase activity in certain tissues (e.g. viscera), haemolymph glucose, and

protein levels of *A. crenata* also increased under both acute and chronic Cd exposure. Overall, the laboratory-based studies clearly demonstrated that the toxicological, biochemical, and physiological responses of *A. crenata* significantly in relation to the Cd exposure concentration, tissue type (e.g. toxicological and biochemical responses) and the duration of exposure. This confirms a potential use of this species as an indicator of trace metal pollution in the environment.

Concentrations of different trace metals (As, Cd, Cu, Ni, Pb, and Zn) in field-collected snails were measured to assess the use of *A. crenata* as a bioindicator for trace metal pollution in New Zealand estuaries. Out of6 trace metals, there were significant correlations between metal concentrations in the sediment and snail soft tissues for Cu ($R^2 = 0.43$, $p < 0.01$), As ($R^2 = 0.36$, $p < 0.05$) and Pb ($R^2 = 0.3$, $p < 0.05$). The sites were distinguished according to the metal content of the snail tissues, metal contamination in sediment, and biomarker responses. Of the biomarkers examined in field-collected snails, those with the greatest promise for detecting environmental impact, related to metal contamination, included the condition index, glutathione-S-transferase, catalase activity, lipid peroxidation, haemolymph glucose, and protein. In addition, this thesis for the first-time evaluated *A. crenata* population attributes (e.g. length and density) as indicators of estuarine pollution using both contrasting areas within and between estuaries. The results indicated significant site-specific and regional differences in snail population structure, and significant positive correlations between sediment total recoverable phosphorous (TRP), to snail density ($R = 0.9$) and shell length characteristics including minimum, mean, and median length to certain sediment trace metals (e.g. Zn and Cd).

The mudsnail, *A. crenata*, can be an effective biomonitoring tool for New Zealand estuaries. As mud snail populations can be used to identify areas that are potentially affected by trace metals and other contaminants. Biomarkers can be used to follow the effects of habitat disturbances, or the recovery of particular areas following habitat remediation, and will provide useful information for environmental managers.

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General Introduction

1.1 Estuarine ecosystems

Estuaries are the most valuable and dynamic natural habitats in the coastal zone and occur where fresh water derived from land runoff is mixed with the seawater (McDowall, 1976). Influxes of high levels of nutrients from the adjacent catchment make estuaries some of the most biologically productive ecosystems in the world (Gonzalez-Dominguez et al., 2016). Estuaries are also important coastal features, both ecologically and with respect to human settlement and use. For example, estuaries play a significant role as nesting, breeding and nursery grounds for many commercially important aquatic species. They also support various human activities such as main transport routes, natural harbours, and tourism (Crawford et al., 1995; Robertson et al., 2002).

New Zealand has more than 350 estuarine systems, ranging from small inlets to large systems, with extensive intertidal and subtidal habitats (Hume et al., 2016). In New Zealand, estuarine ecosystems play a vital role as breeding and nesting grounds for a wide variety of commercially important fish. For example, at least 30 species of commercial fish use New Zealand estuaries at some stage in their life cycle, include yellow-eyed mullet, grey mullet, and yellow-belly flounder, and shellfish species (e.g. cockle, pipis, and shrimp). In addition, they provide a diverse range of unique habitats for many other species including marine worms, reeds, seagrasses, mangroves, algae, birds and marine mammals (Robertson et al., 2002).

1.2 Estuarine pollution

Estuaries are of high ecological, economic and cultural value, but they are often influenced by anthropogenic activities and exposed to continuous influxes of a variety of contaminants including chemicals from industrial waste, agricultural runoff, recreational boating, and organic matter from urban sewage treatment (Melville and Pulkownik, 2006; Marsden and Baharuddin, 2014). The major organic pollutants in estuarine systems include polycyclic aromatic hydrocarbons (PHA), herbicides and organochlorinated pesticides (Cavanagh and Ward, 2014). Although metals are released into the aquatic ecosystems by natural processes such as leaching from the rocks or sediments that rich with marine depositions (Frew et al., 1997; Diaz, 2013), many estuaries are also subjected to high levels of trace metal contamination as a result of urbanization and industrialization. High concentrations of contaminants result in decreased estuarine health

resulting in stress responses in estuarine organisms ranging from the biochemical/physiological to population and community level changes (Funes et al., 2006; Marsden and Swinscoe, 2014; Zhang et al., 2014).

1.2.1 Trace metal pollution

Among estuarine contaminants, trace metals are of particular concern, as they are persistent, non-degradable, and can cause a variety of harmful effects on aquatic organisms (Marsden et al., 2014). These elements normally occur at very low levels in the environment (Chen et al., 2004). Essential trace metals such as iron, copper, zinc, iodine, fluoride, chromium, selenium, manganese and molybdenum are needed by living organisms in small quantities to function properly and are depleted through the expenditure of energy by various metabolic processes. For example, Cu is required for the formation of haemocyanin, the oxygen-transporting pigments in the blood of many aquatic organisms (Taylor and Anstiss, 1999). Also, RNA and DNA polymerase and lactate dehydrogenase are Zn-associated enzymes (White and Rainbow, 1985), but these trace metals can be toxic when they exceed certain threshold levels. Conversely, metals such as Cd, Pb, and Hg are less important with no biological functions. These non-essential metals can be toxic to aquatic organisms even when their environmental concentrations are small (Jakimska et al., 2011) and this may inhibit essential life functions.

Both essential and non-essential trace elements are known to be accumulated by invertebrates including a variety of mollusc species (Phillip, 1977; Marin-Mezquita et al., 1997; Ali et al., 2012). For aquatic organisms, exposed to trace metals, the uptake of those metals occurs via food intake, respiratory pathways and penetration through the skin (Fisher et al., 1996; Shi and Wang, 2005; Jakimska et al., 2011). Waterborne exposure to metals is considered to be primarily mediated through the respiratory pathways (Spicer and Weber, 1991), and penetration through the skin. Nevertheless, total metal concentration in the water does not always correlate with biological effects (Banks et al., 2012). The bioavailability and toxicity of waterborne trace metals to aquatic organisms strongly depends on the chemical speciation of the metals (Fytianos, 2001). In the marine environment, the chemistry of seawater (e.g. high concentrations of ions such as calcium and chloride) acts to reduce trace metal toxicity through the processes of competition and complexation. In the estuarine environment, deposited sediments readily adsorb metals, thus

creating a reservoir of trace metal contaminants (Banks et al., 2012); however, a number of factors are known to affect bioavailability of sediment-bound metals to aquatic biota, including grain size, mineralogy, sedimentation rate, and microbial activity (Deely and Fergusson, 1994; Charriau et al., 2011). Estuaries that are near to industrial and urban areas are likely to contain high concentration of trace metals in the sediments, which may constitute a high risk to estuarine benthic communities (Li et al., 2012). Among the trace metals, copper (Cu), zinc (Zn), nickel (Ni), arsenic (As), cadmium (Cd), and lead (Pb) are of particular concern due to increased concentrations in estuarine and coastal environments resulting from anthropogenic activities.

1.2.1a Arsenic

Arsenic (As) is a non-essential trace metal which is widespread in aquatic environments and considered to be toxic to aquatic organisms even at low concentrations. It is usually found in the environment as inorganic (e.g. arsenic thrioxide, sodium arsenite, calcium arsenate) and organic (e.g. cacodylic acid, methanearsonic acid, phenylarsonic acid) forms and it has been suggested that the inorganic forms of As are the most toxic (Kumari et al., 2017). Arsenic is released into the environment through a combination of natural processes including weathering of bed rocks and volcanic eruptions as well as through a range of anthropogenic activities such as mining activities, combustion of fossil fuel, as arsenical pesticide and herbicides (Smedley and Kinniburgh, 2002). The average concentration of As in seawater is around 1.5 μg L^{-1}, while estuarine systems show wide variation as a result of varying river input, but are typically less than 4 μg L^{-1} (Smedley and Kinniburgh, 2002); however,in coastal areas, subjected to heavy anthropogenic inputs from mining and smelting activities, the As levels can be as high as 1000 μg L^{-1} (Mahar and Butler, 1988). Reported toxic responses in aquatic organisms to As include nuclear abnormalities in mussels, impaired haemocytes lysosomal stability, inhibited activities of glutathione-S-transferase and catalase levels in the bivalve *Lamellidens marginalis* and reduced glutathione peroxidases activity in the mussel *Mytilus galloprovincialis* (Chakraborty and Ray, 2009; Chakraborty et al., 2010, Wang et al., 2014).

1.2.1b Cadmium

Among trace metals, cadmium (Cd) is of significant interest because it is considered one of the most toxic trace metals which poses a serious environment threat (Dabas et al., 2014). This highly toxic, non-essential trace element is distributed ubiquitously in aquatic environments. Cadmium is released into the environment through natural processes such as weathering of Cd-rich geology and volcanism, as well as from anthropogenic sources including mining and metal processing operations, municipal wastewater, fossil fuel combustion and agricultural runoff (Eislar, 1985; Butler and Timperley, 1996). The average concentration of Cd in seawater is around 0.1 μg L^{-1} (Korte, 1983) and sediment concentrations in estuaries can be as high as 400 μg g^{-1} (Griggs and Johnson, 1978). Cd contamination in coastal areas has received significant attention in New Zealand due to extensive use of Cd-contaminated superphosphate fertilisers in near-coastal lowland farms (Butler and Timperley, 1996). Also, run-off from mine tailings may generate waterborne Cd levels as high as 800 μg L^{-1} (Craw et al., 2005). Cd toxicity results in a wide range of toxicological impacts on aquatic organisms including changes in biochemical pathways associated with oxidative stress, altered energy metabolism, impaired calcium homeostasis, perturbed growth and reproduction and at sufficiently high exposure concentrations, death (e.g. Eisler, 1985; McGeer et al., 2012). For example, Cd induced catalase activity was recorded in the brown mussel *Perna perna* (Boudjema et al., 2014). Devi (1996) reported increased in oxygen consumption in Cd-exposed marine bivalve *Mytilopsis sallei*. and exposure to Cd resulted in reduced respiration and ammonia excretion rates in the green-lipped mussel *Perna canaliculus* (Chandurvelan et al., 2012).

1.2.1e Copper

Copper (Cu) is an essential trace metal that is required in small amounts by aquatic organisms, but is toxic to aquatic life at high concentrations (Tchounwou et al., 2012). The largest anthropogenic releases of copper to the environment result from stormwater runoff, mining operations, fertilisers, solid waste and fuel combustion (Cavanagh and Ward, 2014). The average concentration of Cu in seawater ranges from 0.069 – 5.56 μg L^{-1} (Hall and Anderson, 1999). Exposure of aquatic organisms to high concentrations of Cu has resulted in a wide range of toxic responses. For example, reduced lysosomal stability in the intertidal gastropod *Bembicium nanum*, reduced of

glutathione-S-transferase activity in the marine gastropod *Nucella lapillus*, reduced haemolymph protein in the shore-crab *Carcinus maenas***,** reduced lysosomal stability in the limpet *Patella vulgata*, induced metallothionein production in the crab *Carcinus maenas* and (Weeks et al., 1993; Brown et al., 2004; Cunha et al., 2007; Ubrihien et al., 2017).

1.2.1f Lead

Lead (Pb) is a non-essential trace element which is widely distributed and mobilised in the environment. It is released into the environment through natural processes such as weathering of soil, forest fires and volcanic eruptions, as well from anthropogenic sources including discharge of ammunition, leaded fuel in light aircraft and the combustion of coal and wood (Eiser, 1988). In seawater, Pb can occur primarily as lead chloride ($PbCl_2$) and lead carbonate ($PbCO_3$). The concentration of Pb in the ocean is in the range 0.001 – 0.014 µg L^{-1} (Flegal and Patterson, 1983). The Pb concentration in estuarine sediment however, can be as high as 2700 µg g^{-1} (Bryan et al., 1992). Pb is toxic to aquatic organisms even at low concentrations (Jakimsk et al., 2011) and causes a wide range of toxic responses including inhibition of enzyme activity in the freshwater pulmonate gastropod, *Bimpharia glabrata*, histopathological damage in the freshwater snail, *Lanistes carinatus*, increased activity of lipid peroxidation, catalase and glutathione-S-transferase in the green mussel, *Perna veridis* and increased catalase activity in the brown mussel, *Perna perna* (Aisemberg et al., 2005; AbdAllah, 2006; Hariharan et al., 2014; Boudjema et al., 2014).

1.2.1g Nickel

Nickel (Ni) is an essential metal distributed ubiquitously in aquatic environments and introduced into the environment by both anthropogenic (e.g. sewage sludge, stainless-steel processing, processing of machinery and household appliances, electroplating, as pigments; Eisler, 1998; Muyssen et al., 2004) and natural sources, such as weathering of bed rocks. Ni occurs in aquatic systems as soluble salts adsorbed on to clay particles or organic matter such as detritus, algae and bacteria (WHO, 1991). Although the concentration of Ni in open seawater ranges from 0.2 – 0.7 µg L^{-1}, concentrations in estuaries can be as high as 10 µg L^{-1} (Eisler, 1998; Muyssen et al., 2004). It has been reported that the toxicity of Ni in aquatic organisms varies considerably according to species and abiotic factors. Reported toxicity responses due to Ni exposure include reduced growth and significantly disrupted Ca^{2+} homeostasis in the freshwater gastropod *Lymnaea stagnalis*,

glutathione peroxidase activity in the mussel *Mytilus galloprovincialis*, decreased oxygen consumption in the fresh water bivalve *Lammellidens marginalis* and decreased total haemocyte count and increased lysosome activity in the Pacific abalone *Haliotis discus* (Tsangaris et al., 2008; Andhale and Zambare, 2012; Niyogi et al., 2014; Min et al., 2015).

1.2.1h Zinc

Zinc (Zn) is also an essential element for the growth and life cycles of organisms and constitute of more than 200 metalloenzymes (Eisler, 1993), but is toxic at high concentrations. It is used commercially primarily in galvanised metals and metal alloys, but also has wide applications as organic petrochemicals, fertilisers and chemical intermediates (Cavanagh and Ward, 2014). Toxic responses in aquatic organisms, due to elevated levels of Zn, include a significant reduction of acid phosphatase activity and reactive oxygen species production in haemocytes in the marine gastropod *Haliotis tuberculata*, significant reduction of glutathione-S-transferase in the freshwater pulmonate snail *Lymnaea luteola*, decreased net energy budget in the freshwater gastropod *Melanoides tuberculate*, inhibition of oxygen consumption and decreased osmotic pressure in the marine white shrimp *Litopenaeus vannamei*, and induction of metallothionein in the freshwater bivalve, *Corbicula fluminea* (Wu and Chen, 2004; Marie et al., 2006; Moolman et al., 2007; Mottin et al., 2010; Ali et al., 2012).

1.2.2 Nutrient pollution

Nutrients in aquatic systems are the substances that are essential for the growth of algae and aquatic plants. The main nutrients in waterways come in the form of inorganic nutrients such as nitrogen (N) and phosphorous (P) (Smith et al., 1999). Worldwide nutrient inputs into estuarine waters are increasing, and these may lead to the prevalence of eutrophication and hypoxic episodes in these systems. A range of environmental conditions are likely to affect estuarine eutrophic and hypoxic conditions including the presence of other contaminants, biological and physiological properties and sediment geochemistry (Banks, 2011). Increased nutrients can also alter ecosystem function as elevated concentration of nutrients may result in faster growth of plants and algae, reduce water clarity, change sediment chemistry and loss of benthic biodiversity (Fry et al., 2011). High input of nutrients can affect the trophic condition of the estuary, resulting in very high algal growth and then poor oxygen conditions as the algae respire and decay (Zeldis and Whitehead, 2017).

Persistent eutrophic conditions can cause long-term population level changes in some organisms, particularly animals that live in the intertidal zone of the coastal habitats (Grilo et al., 2012; Cardoso et al., 2013; Marsden and Baharuddin, 2014). In New Zealand, intense agricultural and farming activities have significantly increased the concentrations of nitrogen (N) and phosphorus (P) in freshwater flowing into the estuaries, exacerbating eutrophication, harmful algal blooms and subsequent habitat loss (Cavanagh and Ward, 2014).

1.3 Environmental monitoring

Environmental monitoring generally includes measurements of the chemical concentrations in sediment or water (Hanson et al., 2013); however, chemical measurements provide only a snap shot rather than an ongoing assessment of contaminant exposure. Moreover, it is not possible to monitor a large number of substances, as the cost and time soon become unmanageable. Therefore, as an alternative to chemical monitoring, biomonitoring has been used effectively to assess environment contaminants . This is because in some animal species, trace elements can accumulate in tissues according to availability of those contaminants in the habitat (Rainbow, 2002, Marsden and Baharuddin, 2014). Living organisms that can be used to monitor the health of the natural ecosystems in the environment are known as "Bioindicators" (Parmar et al., 2016). The study of organisms as pollutant indicators has several advantages over the chemical analysis of abiotic compartments. Organisms only accumulate the biologically available forms of the pollutant and are always present in the environment, thus enabling the continuous monitoring of pollutants (Marcovecchio, 2004). Another obvious advantage of using animals is that they provide an integrated response to pollutants (Hook et al., 2016; Rossi et al., 2016)

1.4 Bioindicators

Bioindicator species should be relatively sedentary, so that the results can be linked to local areas. They should be large and present in high abundance in order to provide enough tissue for analyses and should be widely distributed to facilitate comparison (Bellante et al., 2016). Furthermore, they must be long-lived to allow for long-term studies, should be robust, and also they should be easy to collect and handle. Invertebrate bioindicators are increasingly used to assess the contaminant load in marine ecosystems (Rainbow et al., 2002). For example, mussels are used worldwide to

evaluate the contaminants present in open coastal habitats (Cantillo, 1998; Chandurvelan et al., 2015); however, a variety of species from different taxonomic and feeding groups including amphipods, cockles and clams have been successfully used to detect trace metal contaminants in estuarine habitats in Europe and New Zealand (Bryan and Hummerstone, 2009; Marsden et al., 2003; Marsden and Swinscoe, 2014).

1.5 Biomarkers

Biomarkers are measures of sub-organismal responses in organisms or exposed biological systems, which can demonstrate exposure to, or the effects of, environmental contaminants (Peakall et al., 1992). Biomarker responses include biochemical responses as well as physiological and histopathological alterations (Shugart, 1996). They provide information on spatial and temporal changes in the concentration of contaminants and indicate environmental quality or occurrence of adverse ecological consequences (Au, 2004). Biomarkers can be considered as a shortcut where the mode of action itself is monitored, rather than monitoring all the chemicals that have that particular mode of action. Thereby, the number of monitored parameters can be significantly reduced.

Biomarkers can be classified into three main groups. The "biomarkers of exposure" reflect an early response to contaminants (Silins and Hogberg, 2011). The "biomarkers of effect" reflect physiological or biochemical changes as a consequence of exposure, while "biomarkers of susceptibility" indicate the natural characteristics (e.g. genes) of the organism which could be influence by a specific agent or toxin, although these have not been developed or incorporated in ecological assessment frameworks (Hook et al., 2014).

In New Zealand, biomarker studies have become an increasingly popular tool to detect exposure and adverse effects of contaminants on aquatic organisms. For example, Chandurvelan et al. (2015) studied biochemical, physiological and immunological responses of the New Zealand green-lipped mussel, *Perna canaliculus* exposed to coastal metal pollution and concluded that this species can potentially use as a bioindicator of coastal metal pollution in New Zealand. McRae et al., (2017) studied biomarker responses in galaxiid fish, *Galaxias maculatus* exposed to anti-inflammatory drug diclofenac and found a wide range of toxicological impacts (e.g. elevated levels

of catalase activity and lipid peroxidation in fish liver) and bioaccumulation of diclofenac in fish tissues. Another study Reed et al., (2011) investigated biomarker responses including metallothionein, glutathione-S-transferase, aldehyde dehydrogenase and Cu/Zn superoxide dismutase in New Zealand cockle, *Austrovenus stutchburyi* and yellowbelly flounder, *Rhombosolea leporine* exposed to estuarine trace metal pollution.

1.6 Gastropods as indicators

Many gastropod species satisfy the criteria required for a potential bioindicator and have been regularly used as contaminant bioindicators to assess the environmental health of coastal and freshwater ecosystems (Table 1.1). They have a wide geographic distribution, are abundant, easy to collect and handle, have a sedentary lifestyle, and are tolerant of a wide range of environmental conditions (Rainbow, 1995; Marsden and Baharuddin, 2014). Gastropod species are widely used as metal indicators because they accumulate trace metals according to the availability of those metals in the environment (Chandran et al., 2005; Reategui-Zirena et al., 2017). In addition, several studies also investigated sensitivity of those organisms to elevated levels of nutrients and found wide range of responses including changes in growth rate, population density variations and migratory responses (Marsden and Baharuddin, 2014; Coffin et al., 2018). Deposit-feeding gastropods have been successfully used as biomonitor species in estuaries where they are dominant members of the macrofauna, occur in high densities and provide linkage between sediment microphytobenthos and animals high up the food chain (Curtis and Kinley, 1998; Antonio et al., 2010; Marsden and Baharuddin, 2014). Moreover, deposit-feeding gastropods that live in estuaries are often exposed to higher levels of contaminants than those in open coastal habitats because they are exposed not only water borne toxins, but they may also be closely associated with contaminated sediments (Marsden and Swinscoe, 2014).

While the sensitivity of terrestrial and freshwater gastropods to different environmental contaminants were well studied under laboratory conditions (Coeurdassier et al., 2003; 2004; Chandran et al., 2005; Li et al., 2008; Ali et al., 2012), the literature on the sensitivity of marine or estuarine gastropods to environmental contaminants is sparse. Although gastropod species have been regularly used as contaminant indicators, these researches have been mainly restricted to few specific areas such as a single estuary (Marsden and Baharuddin, 2014), mangrove areas

(Satheeshkumar and Khan, 2012) or rocky shores (Suratissa and Rathnayake, 2017) and the research to date has not considered biological responses of gastropods to contaminants within and amongst sampling sites. There are studies on mussels (Chandurvelan et al., 2012; 2015) that used multiple biomarker responses to specific contaminants (e.g. metals) in the laboratory and filed. However, it is unaware any published reports that used similar approach to assess estuarine gastropods to specific environmental contaminants. Although gastropods meet the general requirements needed as potential bioindicators, there have been few attempts to validate biomarker responses using field collected specimens and thus enable them to be used in environmental monitoring. In addition, large scale natural and catastrophic events like earthquakes can cause adverse impacts on estuarine organisms. Amongst, intertidal communities gastropods are some of the organisms mostly affected by such catastrophic events and may display a wide range of individual through population level changes. However, it is not surprising that there are only limited studies addressing the impacts of such events on estuarine organisms.

Table 1.1 Biomarker responses of gastropod species from different aquatic habitats exposed to different type of contaminants.

Organism	Natural habitat	Exposure, and duration	Organ studied	Biological effect	Reference
Lymnaea luteola L.	Freshwater	ZnO nanoparticles (24 and 96 h)	Digestive gland	Reduced glutathione, glutathione-S-transferase and glutathione peroxidase, Increased malondialdehyde and catalase	Ali et al., 2012
Gibbula umbilicalis	Marine	Mercury chloride (96 h)	Whole animal	Increased glutathione-S-transferase	Cabecinhas *et al*., 2014
Lymnaea stagnalis	Freshwater	Cu (96 h)	Whole animal	Reduced ammonia excretion, net Ca^{2+} uptake	Brix et al., 2011
Lymnaea stagnalis	Freshwater	Pb (30 d)	Whole animal	Reduced growth and shall formation and Ca2+ uptake	Grosell et al., 2006
Helix aspersa	Terrestrial	Zn, Cu, Pb and Cd (120 d)	Whole animal	Reduced food consumption and fecundity	Laskowsk and Hopkin, 1996
Helix pomatia	Terrestrial	Cd (10 d)	Whole animal	Increased mortality and metallothionein production, Programmed cell death	Chabicovsky et al., 2004
Achatina fulica	Terrestrial	Cd and Zn (48 h)	Digestive gland and kidney	Reduced superoxide dismutase, catalase and glutathione peroxidase	Chandran et al., 2005
Bellamya bengalensis	Freshwater	Hg and Zn 24 to 96 h)	Whole animal	Reduced oxygen consumption	Londhe and Kamble, 2013
Onchidium struma	Marine, Estuarine	Cu (96 h)	Hepatopancreas and muscle	Increased superoxide dismutase and catalase in hepatopancreas	Li et al., 2009
Nucella lapillus	Marine	Cu and Cd (96 h)	Whole animal	Increased activity of cholinesterases by Cd Reduced glutathione-S-transferase by Cu	Cunha et al., 2007
Hinia reticulata	Marine	Tributyltin (1.5 y)	Whole animal	Imposex	Stroben et al., 1992
Hydrobia ulvae	Marine	Eutrophication	Population	Increased density	Cardoso et al., 2002*
Amphibola crenata	Marine	Nutrients	Population	Increased density and scope for growth	Marsden and Baharuddin, 2014*
Buccinanops globulosus	Marine	Tributyltin (TBT)	Whole animal	Imposex	Primost et al., 2016*
Gastropods	Marine	Sewage and domestic effluents	Population	Reduced diversity and density	Suratissa and Rathnayake, 2017*

h -hour, d – day, y – year, ***** Filed studies

1.7 The study organism, *Amphibola crenata* (mud snail)

The endemic pulmonate gastropod, *Amphibola crenata,* is widespread throughout New Zealand, occurring on mudflats of almost all estuaries, and therefore potentially exposed to contaminants from human induced activities including industrial contaminants, urban development and land run off (Shumway, 1981; Little et al.,1984). This deposit feeding gastropod is a dominant member of the intertidal macrofauna and occurs in high densities. Adult *A. crenata* feed on microbenthic algae, bacteria and organic detritus, while newly settled veligers feed predominantly on bacteria (Junipter, 1982; Pilkington and Pilkington, 1984). It provides essential ecological services, as a food source for estuarine predators (e.g. flounder and mullet), provide linkage between sediment microphytobenthos and organisms higher up in the food chain and assists bioturbation (Orvain et al., 2004; Marsden and Baharuddin, 2014). It is long-lived, can tolerate a wide range of environmental conditions, is relatively sedentary and occurs in both clean and contaminated areas (Shumway and Marsden, 1982; Rainbow, 1995; Bennington, 1979). *Amphibola crenata* has been previously proposed as a potential indicator of environmental health conditions of New Zealand estuaries (Marsden and Swinscoe, 2014).

1.8 *Amphibola crenata* as a bioindicator

Few studies have assessed the suitability of *Amphibola crenata* as a bioindicator species. Marsden and Baharuddin (2014) studied growth and survival of *A. crenata* as bioindicators of stress associated with high nutrients and found increased adult growth rate and juvenile survival with improvements in water quality and less microphytobenthic biomass. This finding suggested that gastropod growth and survival could be used as tools to monitor the effects of changing nutrient levels and recovery from eutrophication within temperate estuaries. There are few general trends regarding the effects of eutrophication on grazing gastropods with some studies suggesting that species increase growth in response to increase in inorganic nutrients, whilst in others, there is a decrease in growth rate (Cardoso et al., 2002; Morton and Chan, 2004; Johnson, 2011; Cardoso et al., 2013). Another study on *A. crenata,* reported that the growth of *A. crenata* was different between clean and contaminated sites and that the highest growth occurred where the sediment

and aquatic nutrient levels were highest (Marsden and Swinscoe, 2014). Further, Marsden and Baharuddin (2014) noted a negative correlation in condition index of large *A. crenata* with sediment nutrients and trace metals and suggested that condition index might be a suitable reflection of stressors in *A. crenata*.

1.9 An outline of the biological endpoints used in the study

Numerous biological endpoints have been used to assess the effects of contaminants on aquatic organisms (McCarty and Munkittrick, 1996; Ubrihien et al., 2017). These include population abundance, modal length group, sex ratios, growth rates, and maximal body size (Marsden, 2002; Shriver et al., 2002). More recently, molecular, behavioural, physiological, and biochemical biomarkers have been evaluated as indicators of stress in aquatic organisms (Stroben et al., 1992; Boldina-Coscqeric et al., 2010; Ali et al., 2012; Besser et al., 2016) and assess the health status of aquatic ecosystems. The present study selected physiological biomarkers which indicate the fitness of a species and reflect stress caused by the presence of contaminants (Lagadic et al, 1994). Biomarkers which reflected the energy demands, homeostatic mechanisms and those reflecting oxidative stress pressure were selected. The physiological indices for aquatic organisms included oxygen consumption, ammonia excretion, oxygen to ammonia (O:N) ratio and the body condition index (CI).

Biochemical biomarkers can provide information on stress responses and molecular mechanisms which underlie toxicity (Walker, 1995), and they are likely to be the most sensitive indicators of stress (Thaker and Haritos 1989). Therefore, in the present study, biochemical responses including catalase activity, glutathione-S-transferase (GST), lipid peroxidation, haemolymph metabolites and tissue glycogen in *A. crenata* in relation to the trace metal and nutrient pollution were investigated. The study further assessed population structural variations and reproductive performance of *A. crenata* as an endpoint of effect of contaminants (Table 1.2). The above-mentioned biomarkers were selected in the present study to provide comprehensive and biologically relevant information on the stress effects of an estuarine snail when exposed to contaminants.

Table 1.2 Biomarker responses of *A. crenata* investigated in the present study.

BIOMARKERS			
Physiological	**Biochemical**	**Behavioural**	**Chemical**
• Oxygen consumption • Ammonia excretion • O:N ratio • Condition index	• Catalase • Lipid peroxidation • Glycogen • Haemolymph glucose • Haemolymph protein • Glutathione-S-transferase	• Density • Length • Reproductive performance	• Metal analyses ➢ Tissue ➢ Water ➢ Sediment

- **Physiological biomarkers**

Animal oxygen consumption describes the respiratory capacity and provides an estimate of metabolic rate (Salin et al., 2015). This is combined with the nitrogen excretion rate, mainly ammonia, to describe the relationships between respiratory dependence and utilised resources (carbohydrate and lipid) with the deamination of amino acids (Schmidt-Nielsen, 1997). Ammonium is one of the final products following catabolism principally of amino acids. In addition to being utilised as energy substrates and components of body structures, amino acids can be more important than ions in the maintenance of osmotic pressure in animals (Gilles, 1972; Rosas et al., 1999). Normally, increases in ammonium excretion reflect an increase in catabolism of amino acids or other nitrogenous compounds, indicate that organism already started to utilise it protein reserves as an energy source to meet energy demand under stressful environmental conditions.

The rate of oxygen uptake: nitrogen excretion (O:N) is a good indicator of metabolic shifts and the amount of energy available. Study on the effects of trace metals on the respiration of decapod crustacean have demonstrated that decreased oxygen consumption rates were related to metal concentration and exposure time (Barbieri, 2011).

The condition index (CI) reflects variations in the physiological state of an organisms. It is a simple and frequently used biomarker for aquatic organisms to evaluate nutritional status and physiological stress (Wilson, 1988; Norkko and Thrush, 2006; Marsden and Baharuddin, 2014). It also reflects overall health of an ecosystem (Damiens et al., 2007). The CI varies with the food availability, quality of the diet and natural or anthropogenic stresses (Bodin et al., 2004; Tsangaris et al., 2010; Marsden and Baharuddin, 2014).

- **Behavioural biomarkers**

Environmental stressors can affect invertebrate populations, and ecological indicators such as population structure and reproduction are recognised as non-specific bioindicators (Marsden and Swinscoe, 2014). These indicators provide an integrated response to multiple stressors and reflect ecosystem structure and function (Marsden, 2002; Shriver et al., 2002; Kim et al., 2003). A number of studies have evaluated reproductive success of key aquatic species as a potential indicator of stress, because aquatic pollutants are known to adversely affect on different reproductive life-stages of aquatic organisms and may ultimately result in population level changes (e.g. abundance and distribution) (Woin and Bronmark, 1992; Ortiz-Zarragoitia and Cajaraville, 2006; Pang et al., 2012).

- **Biochemical biomarkers**

A commonly reported effect of metal toxicity in organisms is oxidative stress (Stohs et al., 2001). In animal tissues, oxidative stress can be measured using a number of different endpoints, most commonly markers of oxidative damage (e.g. lipid peroxidation), or changes in oxidative defense mechanisms (e.g. enzymes such as catalase that convert hydrogen into water and oxygen). An increase in lipid peroxidation has been reported in mussels exposed to Cd (e.g. Pytharopoulou et al., 2011). There are, however, other reports of high, acute waterborne Cd exposures causing no increase in tissue lipid peroxidation (e.g. green-lipped mussel; Chandurvelan et al., 2013). Glycogen level is one of the parameters that reflects the energetic and reserves status of an organism (Ansaldo et al., 2006). Moreover, glycogen is used rapidly when some organisms are under stress, and levels of this energy reserve have been suggested as useful biomarker of general

stress (Leung and Furness, 2001; Vasseur and Cossu-Leguille, 2003; Ansaldo et al., 2006;). In gastropods, such as the snail *Biomphalaria glabrata*, glycogen is considered to be the principal energetic reserve, and is found in specific storage cells, which are widely distributed in the whole body of the snail (Geraerts, 1992).

Glutathione-S-transferases (GST) are a group of antioxidant defense enzymes present in aquatic organisms where the main function is detoxification of different xenobiotic compounds such as trace metals (Cunha et al., 2007). The GST enzymes protect cells against toxicants by conjugating the thiol group of the glutathione to electrophilic xenobiotics. Several studies have reported induction of GST activity in aquatic organisms including snails, mussels and fish species when exposed to anthropogenic stressors (Looise et al., 1996; Hamed et al., 2003; Hansson et al., 2006). Therefore, GST is proposed as a potential biomarker of environmental contamination.

In recent years, haemolymph metabolites such as protein and glucose have been identified as indicators of physiological condition in organisms exposed to different environment conditions (Carefoot, 1994; Rosas et al., 2004; Rosas et al., 2007). The haemolymph glucose concentration in aquatic organisms has been recognized as a potential biomarker of a variety of anthropogenic stressors such as trace metals, anoxia and elevated CO_2 (Hall and Van Ham, 1998; Lorenzon, 2005; Hannan et al., 2016). Glucose in animal haemolymph can be derived from stored glycogen (Cameselle et al., 1980) or exogenous sources such as diet (Silva and Wright, 1992). Lorenzon et al. (2000) reported elevated haemolymph glucose in the shrimp, *Palaemon elegans* exposed to sublethal concentrations of Hg, Cd and Pb. A number of studies however, have shown that the effects of anthropogenic stressors on haemolymph glucose may vary greatly depending on the species and type of the stressor (Lorenzon et al., 2000; Bislimi et al., 2013). Haemolymph proteins play vital roles in animal physiology, transporting O_2, maintaining osmotic pressure, assisting reproduction and inducing stress responses (Lorenzon et al., 2011). Chandurvelan et al. (2013) reported that exposure of green-lipid mussel, *Perna canaliculus*, to subchronic (21 days) Cd exposure, resulted in an increase in haemolymph protein levels, suggesting that the mussels had started to utilise proteins as an energy source.

1.10 Research objectives

The aim of this study was to assess, the use of the mudflat snail, *Amphibola crenata* as a potential bioindicator for anthropogenic and natural stressors using physiological, biochemical, and population-level biomarker responses. The objectives of this study were to:

1) Investigate the acute (48 h) and chronic (21 d) toxic effect of Cd in *A. crenata* using multiple biomarkers. These laboratory-based studies were undertaken to understand toxic mechanisms and sensitivity of this species to metal contaminants.

2) Conduct a field study to evaluate the suitability of *A. crenata* as a potential bioindicator for trace metal and nutrient pollution in estuarine ecosystems.

3) In addition, the study assessed biological responses of *A. crenata* to large-scale disturbances associated with the earthquake events.

The primary hypothesis of this research was that anthropogenic and natural stressors would significantly impact cellular, organismal and population-level aspects of the biology and ecology of *Amphibola crenata*. To test this hypothesis, individuals were sampled from both contaminated (e.g. trace metals and nutrient) and less or non-contaminated areas within selected estuaries in the South and North Islands of New Zealand. The research used both field-based and laboratory investigations. Further, in order to examine responses of *A. crenata* to natural and catastrophic disturbances such as earthquakes, population attributes were compared with post-earthquake records (2008/2009 study by Nursalwa Baharuddin).

1.11 Thesis outline

This thesis is organised into seven chapters. The first chapter (introduction) provides an overview of the trace metal and nutrient pollution in estuarine ecosystems worldwide. It also includes a review of gastropod species used as bioindicators to monitor various type of contaminants.

Chapter 2 examines the physiological and biochemical responses of *A. crenata* to acute (48 h) waterborne Cd toxicity. This study assessed Cd toxicity to *A. crenata* under an acute time frame.

Chapter 3 describes the effect of chronic (21 d) Cd toxicity on *A. crenata* physiology. The study tested the effect of dose- and time-dependent physiological changes in *A. crenata* under chronic exposure.

Chapter 4 focuses on *A. crenata* as a bioindicator of both anthropogenic activities (e.g. trace metal and nutrient pollution), and large-scale catastrophic events such as earthquakes. In this study, snails were collected within the same estuary (Avon-Heathcote/Ihutai) from contrasting areas with different levels of anthropogenic pressure to assess site-specific variations in *A. crenata* attributes. The study also compared the mud snail's population attributes and sediment characteristics obtained from this study with 2a study (post-earthquake) by Nursalwa Baharuddin (2008/2009).

Chapter 5 examines the relationship of *A. crenata* population attributes (e.g. density and length) to sediment characteristics (e.g. trace metals, nutrients and organic content, and particle size distribution).

Chapter 6 discusses the results of a field study conducted using a multiple biomarker approach to evaluate *A. crenata* as a potential bioindicator for trace metal pollution in New Zealand estuaries.

Chapter 7 presents an overall synthesis and general discussion of feasibility of using *A. crenata* as a bioindicator for anthropogenic and natural stressors.

Chapter 2 has been submitted to the Journal of Ecotoxicology and Environment Safety as a manuscript and the Chapter 4 was submitted to the Avon-Heathcote/Ihutai Trust as a report.

Chapter 2

Acute waterborne cadmium toxicity in the estuarine pulmonate mud snail, *Amphibola crenata*

2.1 INTRODUCTION

Estuaries are demanding environments, exposing the biota therein to extreme fluctuations in salinity, dissolved oxygen, temperature, and nutrients (e.g. Hubertz and Cahoon, 1999), all factors that threaten organism homeostasis. Estuaries are also sinks for contaminants, which further challenge health and survival. This is especially true of estuaries located near urban centres, which may receive domestic, industrial and agricultural effluents, either through direct inputs, or via the river systems that drain into them (Matthiessen and Law, 2002). Furthermore, a variety of physical and chemical factors can effectively trap contaminants within estuaries (Ridgway and Shimmield, 2002), exposing estuarine biota to potentially harmful concentrations of a diverse range of toxicants.

Among the contaminants of particular concern in estuarine settings are trace metals. These are environmentally persistent and can cause a variety of toxic effects in the exposed animal (e.g. Chandurvelan et al., 2015). Sub-lethal (e.g. biochemical and physiological) effects can lead to changes in organism health and fitness, eventually resulting in changes at the population, community and ecosystem level (e.g. Lagadic et al., 1994; Marsden and Swinscoe, 2014). Among trace metals, cadmium (Cd) is of significant interest. This highly toxic, non-essential trace elements is distributed ubiquitously in aquatic environments. It is released into the environment by both anthropogenic (e.g. municipal wastewater, fossil fuel combustion, metal processing, agricultural runoff; Eisler, 1985; Butler and Timperley, 1996) and natural sources, such as weathering of Cd-rich geology and volcanism (Eisler, 1985). Although the average concentration of Cd in seawater is around 0.1 µg L^{-1} (Korte, 1983), marine sediment concentrations can be as high as 80 µg g^{-1} (Böning et al., 2004). Estuarine Cd contamination has received significant recent attention in New Zealand (e.g. Chandurvelan et al., 2015; 2016), owing in part to concerns regarding Cd contamination of superphosphate fertilisers applied extensively to near-coastal lowland streams (Butler and Timperley, 1996; McDowell, 2010), and run-off from mine tailings that can result in river Cd concentrations as high as 800 µg L^{-1} (Craw et al., 2005). Cadmium can cause a wide variety of toxicological impacts on aquatic organisms including changes in biochemical pathways associated with oxidative stress, altered energy metabolism, impaired calcium homeostasis, perturbed growth and reproduction, and at sufficiently high exposure concentrations, death (e.g. Eisler, 1985; McGeer et al., 2012).

One group of aquatic organisms that have been reported as displaying high sensitivity to trace metals (Grosell et al., 2006; Brix et al., 2011), including Cd (Das and Khangarot, 2010), are the freshwater pulmonate gastropods. For example, *Lymnaea stagnalis* is highly susceptible to copper toxicity, stemming from impairments in ion regulation that affect shell development (Brix et al., 2011), while inhibition of feeding and growth are observed at Cd concentrations as low as 32 µg L^{-1} in *L. luteola* (Das and Khangarot, 2010). It is, however, important to note that not all studies support the conclusion of high sensitivity of freshwater pulmonate snails to trace metals. A study on copper toxicity comparing pulmonate snails with hydrobiid snails, found that toxicity was similar in both groups (Besser et al., 2016). The difference in the sensitivity of pulmonate snails in this study relative to other work, was attributed to the authors use of a distinct age class of animals (Besser et al., 2016). However, this finding of relatively high tolerance is consistent with Cd toxicity studies in terrestrial pulmonate snails (Chabicovsky et al., 2004). It is intriguing that, irrespective of sensitivity, both freshwater and terrestrial pulmonates have an excellent capacity for Cd bioaccumulation (Chabicovsky et al., 2004; Das and Khangarot, 2010). For example, bioconcentration factors of more than 6000 have been reported in *L. palustris* exposed to 160 µg L^{-1} waterborne Cd for four weeks (Das and Khangarot, 2010). However, it is unaware any published reports that detail the sensitivity of marine or estuarine pulmonate snails to Cd, or that delineate their capacity for Cd bioaccumulation.

In the current study, the mortality, bioaccumulation, biochemical and physiological impacts of waterborne Cd exposure were examined in the estuarine pulmonate mud snail, *Amphibola crenata*. This species is widely distributed throughout New Zealand and is found in both relatively clean and contaminated areas (Marsden and Baharuddin, 2015). It has a sedentary lifestyle, is abundant year-round, easy to collect, and is highly tolerant to extremes of temperature, aerial exposure and desiccation (Shumway, 1981; Shumway and Marsden, 1982). Little is known regarding the sensitivity of this species to metal contaminants, however it has been shown that those snails inhabiting settings with high sediment metals had decreased condition index (Marsden and Baharuddin, 2015).

The studies were conducted in the laboratory under acute exposure conditions. Laboratory-based testing overcomes some of the practical difficulties in determining the effects of individual

stressors in the field, and thus is an essential tool for understanding the mechanisms of contaminant impacts in organisms. An understanding of toxic mechanisms is itself essential for development of predictive models, allowing extrapolation between different species on the basis of shared pathways of impact. Mechanistic data are also increasingly important for the development of regulatory tools (e.g. Biotic Ligand Model), which facilitate site-specific determination of toxicity based on knowledge of water chemistry, and mechanisms of uptake and toxicity (Di Toro et al., 2001). Studies performed over acute time-frames provide rapid and reproducible estimates of the effects of contaminants on biota and are critical to the development of acute-to-chronic toxicity ratios, which are the basis of many water quality criteria (e.g. Shuhaimi-Othman et al., 2013). Moreover, when coupled with measurement of mechanistic endpoints, these tests provide an overview of toxic responses in an organism (Chandurvelan et al., 2012), and may help in the future selection of biomarkers and bioindicator species for environmental monitoring (e.g. Shuhaimi-Othman et al., 2012).

In the current study, biochemical and physiological endpoints were used to characterise the mechanisms by which Cd exerts its effects. These included physiological indices of metabolic impairment such as oxygen consumption, ammonia excretion and the oxygen to nitrogen ratio (O:N), which have previously been shown to be impacted by Cd in aquatic biota (e.g. Barbieri and Paes, 2011; Chandurvelan et al., 2012; 2017). In addition, biochemical correlates of energy use (tissue glycogen, haemolymph glucose) were measured. Biochemical analysis also included measures of oxidative stress, based on the ability of Cd to induce reactive oxygen species (ROS), likely via displacement of redox active metals from cofactor binding sites (Nair et al., 2013). Cadmium has also been shown to impair the function of enzymes that scavenge ROS, and thus generate oxidative damage indirectly (e.g. Chandran et al., 2005; Chandurvelan et al., 2013; McRae et al., 2018). Overall, the objectives of the present study were to determine toxicological, physiological and biochemical responses of *A. crenata* to acute Cd exposure, and to investigate Cd bioaccumulation in different tissues as a function of exposure levels.

2.2 MATERIALS AND METHODS

2.2.1 Sample collection and maintenance

Adult mud snails (>18 mm) were collected during December 2015 from the mouth of the Avon-Heathcote Estuary/Ihutai (S 43˚33.136', E 172˚44.709') in Canterbury, New Zealand. Recently measured sediment Cd concentrations near the site of collection were 0.1 µg g dry weight^{-1} (Chandurvelan et al., 2016). Snails were then transported to the aquarium at the University of Canterbury, where they were washed thoroughly with filtered natural seawater, and attached algae and mud was scraped from the shells to avoid potential negative impacts on Cd concentration of the treatments due to adsorption by mud particles or accumulation into algae cells. Snails were then transferred into a holding tank containing 2 L of 20 ppt filtered natural seawater (pH 7.6; Na 325 mmol L^{-1}, Ca 9 mmol L^{-1}; Mg 37 mmol L^{-1}; K 8 mmol L^{-1}; Cl 360 mmol L^{-1}; dissolved organic carbon 0.3 mg C L^{-1}) made by diluting Lyttelton Harbour seawater with City of Christchurch artesian well water, and acclimated for 48 hours prior to the experiments, under constant temperature (15 ± 0.5°C) and photoperiod (12 hours light: 12 hours dark). Snails were not fed during acclimation.

2.2.2 Determining median lethal concentration (LC_{50})

Toxicity testing was carried out in constantly-aerated acid-washed 1.5 L polypropylene containers at 15°C and 20 ppt salinity (natural SW as described above) at six Cd exposure concentrations (nominally: 0, 8, 16, 32, 64, and 128 mg Cd L^{-1}), achieved by the dilution of a stock solution of 10 g L^{-1} Cd (as $CdCl_2 \cdot 2½H_2O$). Each exposure concentration was replicated 6 times, with 15 individual mud snails per replicate. Snails were assigned randomly to the different treatments, and were not fed during the 48-h exposure period. A 15-mL water sample was taken from each treatment at time 0 and 48 h, by filtering through a Millex 0.45 µm filter (Millipore Ltd, Cork, Ireland), with these samples subsequently acidified to pH < 2 using 70% ultrapure HNO_3. Acidified samples were then diluted 30 x with milli-Q water and stored at 4°C until analysed by Atomic Absorption Spectroscopy (see below). Mortality was assessed every 12-h throughout the experiment period, with death defined as the point when immobile mud snails failed to respond to probing using forceps. The LC_{50} values were calculated based on measured Cd exposure concentrations.

2.2.3 Bioaccumulation, biochemistry, and physiology

Surviving individuals from the 0, 8, 16 and 32 mg Cd L^{-1} exposure concentrations used to determine the LC_{50} were then used for assessment of tissue Cd content, and physiological and biochemical responses to Cd exposure. While these exposure concentrations are significantly elevated relative to those likely to be experienced in natural settings, they provide a means of exploring the mechanisms by which Cd exerts toxic effects on *Amphibola*.

Immediately following the 48-h exposure, two groups of snails were subjected to physiological assays (see below; each n = 6), while a separate group of animals was sampled for tissue Cd bioaccumulation (n = 6) and for biochemical assays (n = 6). Each n value represents an individual snail from a separate exposure concentration replicate, to avoid pseudoreplication. For animals destined for biochemical assays, haemolymph samples were collected from the foot muscle sinus using a 21-gauge needle and syringe. Then for both accumulation and biochemistry endpoints, snails were blotted dry removing any adherent mucus, and dissected into three anatomical regions: gonad and digestive gland, foot muscle, and remaining tissues (i.e. kidney, mantle, remaining digestive tissues and heart). The gonad and digestive gland (hereafter denoted as viscera) were grouped as one tissue due to practical difficulties to separate them completely. All tissue samples were immediately stored at -80°C until further analysis. Where appropriate, all biochemical biomarker responses were expressed in terms of wet tissue weight. For snails subjected to physiological studies, all soft tissues were removed and dried to a constant weight in an oven at 60°C for 3 days. Dissected tissues for Cd analysis were treated similarly, and subsequently all values for physiological biomarkers and tissue Cd content were expressed in terms of dry tissue weight of the mud snails.

2.2.3a Physiological endpoints

Closed system aquatic respirometry was used to measure the rate of oxygen consumption of individual mud snails (n = 6), following a protocol described by Chandurvelan and colleagues (2012). Glass respirometry chambers (100-mL) were filled with oxygen-saturated 20 ppt seawater and maintained in a 15°C water bath overnight. Pre-weighed mud snails were then placed in each chamber, which was sealed tightly with a rubber bung. This bung had a syringe port which

facilitated the sampling of the water. A control chamber without a mud snail was also maintained, to account for any microbial contribution to oxygen consumption. Water samples were taken at time 0 and 1 h and the decline in oxygen partial pressure (PO_2) inside the chamber was recorded using an oxygen electrode (Strathkelvin 1302), which was calibrated before each measurement in oxygen-saturated 20 ppt water and a sodium sulfite solution. The PO_2 was recorded via a PowerLab (ADInstruments, Waverly, Australia) data recording system. The oxygen consumption rate was calculated using following equation (1) and expressed as μmol O_2 g^{-1} h^{-1}:

$$O_2 \ consumption\ rate = \frac{\Delta PO_2 \times C \times V}{W \times t} \quad \text{.....(1)}$$

where, ΔPO_2 is the change in PO_2 inside the chamber, C is O_2 solubility (μmol O_2 L^{-1}) at 15°C and 20 ppt, V is the volume of water corrected for snail mass (L), W is the snail soft tissue dry weight (g) and t is the time (h).

To determine ammonia excretion, a separate group of individual mud snails (n = 6) was transferred into 50-mL plastic containers filled with 20 mL of 20 ppt filtered seawater. Containers were covered with aluminum foil and left for 6 hours at 15°C. A blank without a mud snail was also maintained. Initial and final ammonia concentrations were measured using a salicylate-based ammonia assay (Charan-Dixon et al., 2017). Ammonia excretion (μg g^{-1} h^{-1}) was then calculated using following equation (2):

$$Ammonia\ excretion = \frac{\Delta C_{NH_4^+} \times V}{1000 \times t \times W} \quad \text{.....(2)}$$

where, $\Delta C_{NH_4^+}$ is the change in NH_4^+ concentration (μg L^{-1}), V is the volume of water (L), t is the time (h), and W is the dry weight of the snail soft tissue (g).

The O:N ratio was then estimated from oxygen consumption and ammonia excretion values as follows (3) (Chandurvelan et al., 2012):

$$O{:}N\ ratio = \frac{(O_2\ mg\ h^{-1})/16}{(NH_4^+\ mg\ h^{-1})/14} \quad \text{.....(3)}$$

As oxygen consumption and ammonia excretion were measured in separate animals, only mean values were used.

2.2.3b Biochemical endpoints

Tissue catalase activity was determined by an assay utilising the decomposition rate of hydrogen peroxide (H_2O_2; Chandurvelan et al., 2013; McRae et al., 2016). The tissue samples were homogenised in 800 µL ice-cold homogenisation buffer (100 mM Trizma base, 2 mM EDTA, 5 mM $MgCl_2.6H_2O$; pH = 7). Of this homogenate, 200 µL was used for lipid peroxidation assessment (see below). The remaining homogenate (600 µL) was centrifuged at 30,000 × g for 10 min at 4°C. The resulting supernatant was diluted 30 × with the homogenisation buffer, and the reaction was initiated by the addition of H_2O_2, with the decline in absorbance at 240 nm measured in a microplate reader at 25°C (UV star; Greiner Bio-One). Supernatant protein concentration was measured via a Bradford assay (Bradford, 1976), using a standard curve based on bovine serum albumin. Catalase activity was expressed as µmol mg protein^{-1} min^{-1}.

Tissue lipid peroxidation was measured using a commercial lipid peroxidation assay kit (Sigma Aldrich, MAK085), according to the manufacturer's instructions. This method is based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) to form a coloured product proportional to the MDA present in the sample. The assay was initiated by adding 300 µL of MDA lysis buffer and 3 µL of butylated hydroxytoluene (BHT) to the homogenate (200 µL; see above). This mixture was centrifuged at 13,000 × g for 10 min. To 200 µL of the resulting supernatant, 600 µL of TBA solution was added, with this solution incubated at 95°C for 1 hour. Once samples cooled, 200 µL was transferred to a 96-well plate reader and absorbance was measured at 532 nm. The amount of tissue lipid peroxidation was expressed as µmol MDA mg protein^{-1}, where protein levels were derived from the Bradford assay (see above).

Tissue glycogen concentration was determined by a coupled enzyme assay (Sigma Aldrich, MAK016). The tissue samples were homogenised in ice-cold deionised water and boiled for 5 minutes to deactivate glycolytic enzymes. Samples were then centrifuged at 13,000 × g and the

resulting supernatants were diluted 100 × using deionised water. After adding the hydrolysis buffer and hydrolysis enzyme mix, samples were incubated at room temperature for 30 minutes. The master reaction mix, including development buffer, development enzyme mix and fluorescent peroxidase substrate, was added to each sample and absorbance was measured at 570 nm using a 96-well plate reader. To quantify the glycogen concentration, a calibration curve was performed using a commercially available standard glycogen (Sigma Aldrich). Glycogen level was expressed as mg g wet weight^{-1}.

The glucose concentrations in snail haemolymph were quantified using a commercial glucose assay kit (Sigma Aldrich, GAHK-20), according to manufacturer instructions. Haemolymph protein concentration was measured via the Bradford method (Bradford, 1976) and expressed as mg mL^{-1}.

2.2.4 Water and tissue Cd concentrations

Acidified and filtered seawater samples (pH < 2) taken from the Cd exposures, were diluted 30 × with 2% HNO_3. Diluted samples were then analysed by Atomic Absorption Spectroscopy (AAS, Varian; 220FS) at wavelength of 326.1 nm. To quantify the Cd concentration, a calibration curve was performed using a commercially-available Cd standard (Fluka Analytical). The limit of detection for this approach was 1 μg L^{-1}. The reported measured Cd exposure concentrations represent the mean values of initial and final water samples for each exposure replicate, which were then averaged across all replicates. Measured Cd concentration data were then subjected to Cd speciation analysis to determine the concentration of bioavailable Cd (i.e. Cd^{2+}). This analysis was performed using the water chemistry described in Section 2.1 and the Visual MINTEQ geochemical modelling program (ver. 3.1; Gustafsson, 2012).

Cadmium bioaccumulation in snail tissues was determined using inductively coupled plasma mass spectrometry (ICP-MS, Agilent-7500cx), using a modification of the method described by Chandurvelan et al. (2012). Dried tissue samples were acid digested overnight using 0.25 mL of 70% ultrapure HNO_3, before being heated at 85°C for 1 h. The samples were diluted 5 × with 2% HNO_3 and analysed by the ICP-MS for Cd. Quality assurance/quality control was achieved by analysing blanks and replicates (n = 2) of a certified mussel tissue standard reference material

(SRM 2976; National Institute of Standards and Technology, US). The mean ± SD recovery of the SRM for Cd was 89 ± 8%. Tissue Cd concentration was expressed as μg g dry wt.$^{-1}$. The limit of detection for tissue Cd using this method was 0.02 μg g^{-1}.

2.2.5 Statistical analyses

All data were processed using R statistical software (R version 3.0.2). Median lethal concentration (LC_{50}) and 95% confidence interval values for *A. crenata* were calculated using probit analysis. For tissue Cd bioaccumulation, catalase activity, lipid peroxidation and glycogen levels, significant effects of Cd treatment, tissue type, and the interaction between these two factors, were determined with a two-way ANOVA followed by a Tukey's post hoc test. All other physiological and biochemical responses were analysed via one-way ANOVA followed by post hoc Tukey's test. All data were tested for, and passed, normality and homogeneity of variance assessments using Shapiro–Wilk and Fligner–Killeen tests before being analysed by parametric analyses. Regression analysis was used to determine the relationship between Cd bioaccumulation and Cd exposure concentrations, using either a linear or hyperbolic curve-fit and applied to individual, not mean, data. The relationship which fitted the data the best (i.e. highest R^2 value) is reported. The goodness of fit of a linear relationship between biochemical/physiological endpoints and Cd exposure concentration (for haemolymph measures and physiological endpoints) or tissue Cd concentration (for measures in viscera, foot or remaining tissues) was also determined. All data, except O:N ratio (overall mean value only; see Section 2.3.1), are presented as mean ± SEM, unless otherwise stated. A value of $p < 0.05$ was considered statistically significant.

2.3 RESULTS

Table 2.1 presents the nominal and measured concentrations of Cd for each exposure treatment. These data show that measured values were close to nominal concentrations, and that there was little variation between replicates. In all concentrations, 6.6% of Cd was present in the dissolved ionic form (Cd^{2+}).

Table 2.1 Nominal Cd measured Cd, and predicted Cd^{2+} (Visual MINTEQ) concentrations in exposure solutions. Nominal and measured values are expressed as mean ± SD of 6 replicates.

Nominal concentration (mg Cd L^{-1})	Measured concentration (mg Cd L^{-1})	Predicted Cd^{2+} concentration (mg Cd^{2+} L^{-1})
0	0.033 ± 0.01	0.002
8	8.83 ± 0.31	0.58
16	16.77 ± 0.51	1.11
32	30.79 ± 1.03	2.03
64	61.00 ± 1.99	4.03
128	119.37 ± 3.68	7.88

No mortality occurred in control animals throughout the experiment. Mortality first occurred at a waterborne Cd concentration of 16 mg L^{-1}, with complete mortality observed at 128 mg Cd L^{-1}. The 48-h median lethal concentration (LC_{50}) of *A. crenata* for Cd was 50.4 mg L^{-1} with lower and upper 95% confidence limits of 44.1 and 56.7 mg L^{-1}, respectively. When based on the predicted Cd free ion (Cd^{2+}) concentration, the 48-h LC_{50} value was 3.33 mg L^{-1}.

The tissue Cd concentration of the control mud snail group was low (< 0.8 µg g dry $wt.^{-1}$) in all tissues. However, in snails exposed to Cd, increases in tissue Cd burden were observed, with a two-way ANOVA showing significant effects of Cd exposure concentration ($p < 0.01$), tissue ($p < 0.01$), and also a significant interaction between these two factors ($p < 0.01$; Figure 2.1). In all Cd exposure concentrations, the viscera (gonad and digestive gland) displayed a significantly higher Cd accumulation than that found in the foot muscle and remaining tissues (i.e. kidney, mantle, remaining digestive tissues and heart). However, in the viscera and the foot, there was no significant increase in tissue burden when increasing Cd exposure concentration from 8 to 32 mg L^{-1}. This is in contrast to the "remaining tissues", where a significant increase in tissue Cd was

seen at 32 mg Cd L^{-1}. The relationship between Cd exposure concentration and tissue Cd accumulation was best described by a hyperbolic relationship for viscera ($R^2 = 0.645$, $p < 0.001$) and remaining tissues ($R^2 = 0.811$, $p < 0.001$), and a linear relationship for foot muscle ($R^2 = 0.822$, $p < 0.001$; Table 2.2).

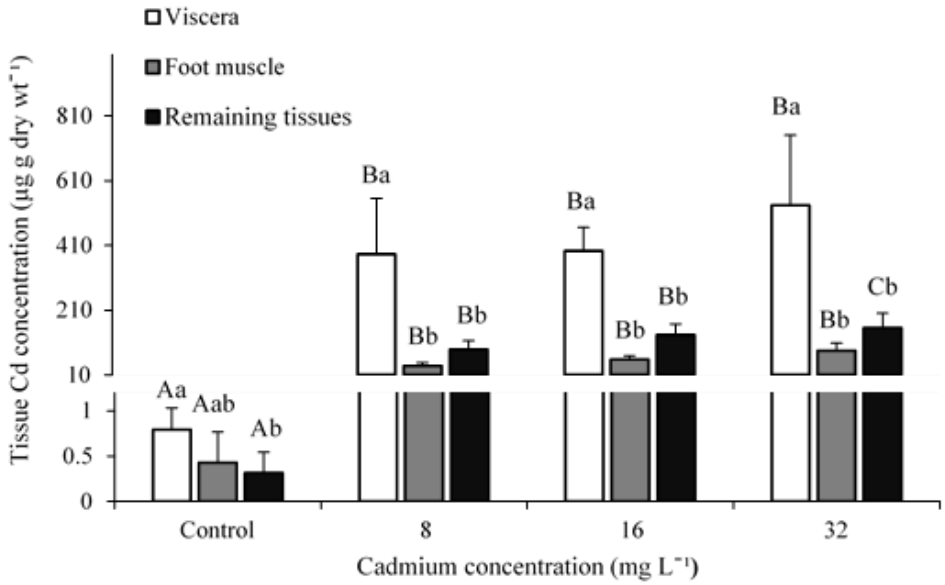


Figure 2.1 Cd accumulation in viscera, foot muscle, and remaining tissues of *Amphibola crenata* following an acute 48-h waterborne exposure. Plotted values represent mean ± SEM of 6 replicates. Bars sharing lowercase letters are not significantly different with respect to tissue type within an exposure concentration, while bars sharing uppercase letters are not significantly different between different exposure concentrations, within the same tissue, as determined by two-way ANOVA followed by Tukey post-hoc test at $p < 0.05$.

Table 2.2 Relationship between exposure Cd concentration and bioaccumulation of Cd in different tissues of mud snails.

Tissue	Coefficient of determination (R^2)	Equation for the line best fit	p - value
Viscera	0.645	$y = (-0.69x)/37.4 + x)$	< 0.001
Foot muscle	0.822	$y = 2.7x + 7.7$	< 0.001
Remaining tissues	0.811	$y = (-0.2x)/(11.6 + x)$	< 0.001

Relative to the control, oxygen consumption rates for *A. crenata* exposed to 8, 16 and 32 mg Cd L^{-1} decreased significantly by 56, 76 and 92% ($p < 0.05$; Figure 2.2A). There were, however, no significant differences in oxygen consumption between Cd-exposed snails. Ammonia excretion increased significantly (by 68 and 84%, respectively) in animals exposed to 16 and 32 mg Cd L^{-1} ($p < 0.05$; Figure 2.2B). The responses of both of these physiological endpoints were strongly and significantly correlated to Cd exposure concentrations (oxygen consumption: $R^2 = 0.659$, $p < 0.01$; ammonia excretion: $R^2 = 0.576$, $p < 0.01$; Table 2.3). Determination of oxygen consumption and ammonia excretion allowed the calculation of the O:N ratio for *A. crenata*. These data showed that molar ratio of oxygen consumed to ammonia excreted declined with increasing Cd exposure concentration, with values of 17, 7 and 2 reported at Cd exposure concentrations of 8, 16 and 32 mg Cd L^{-1}, respectively. The O:N in control conditions was 50 (Figure 2.2C). As oxygen consumption and ammonia excretion were determined in separate snails, these data represent group mean values, and were not able to be statistically compared.

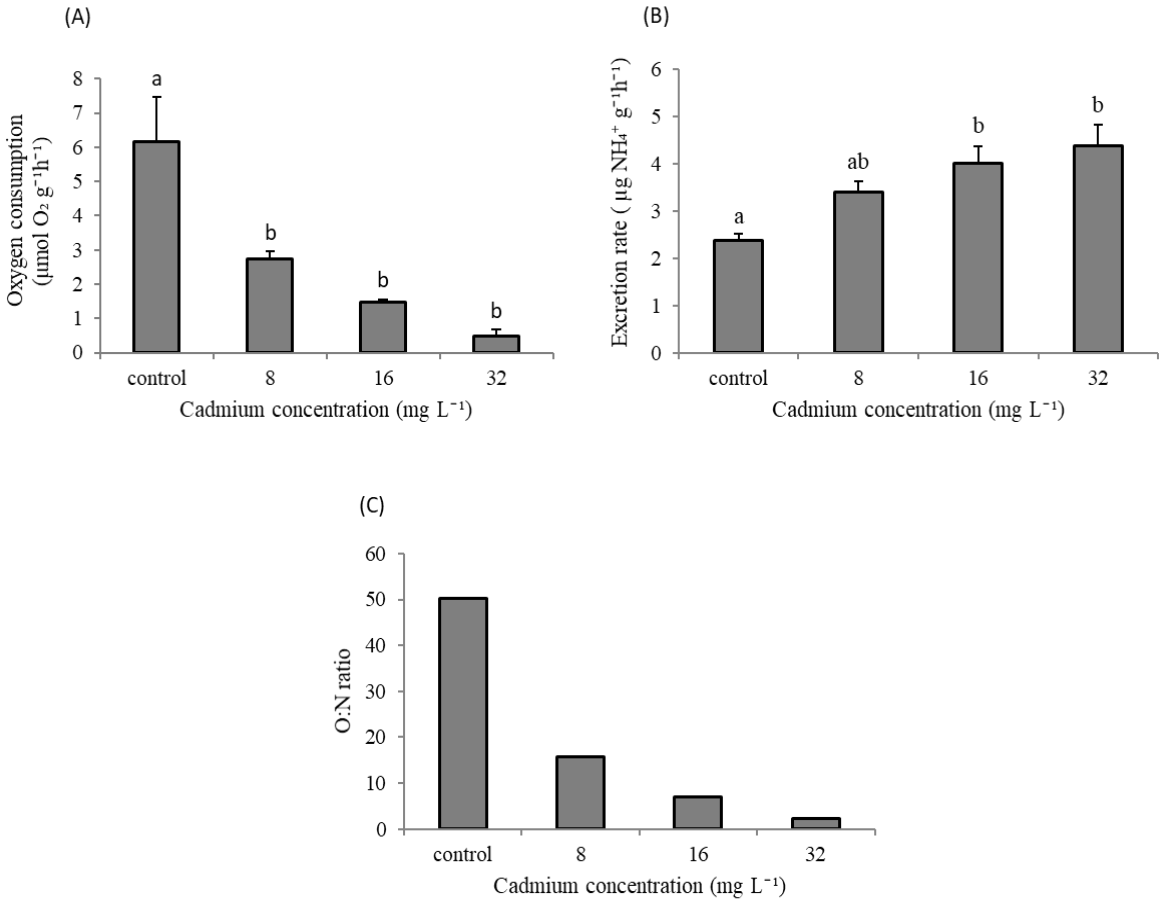


Figure 2.2 The effect of acute (48 h) waterborne Cd exposure on *A. crenata* oxygen consumption (A); ammonia excretion (B); and O:N ratio (C). Plotted values in (A) and (B) represent mean ± SEM of 6 individuals, while plotted values in (C) represent O:N ratio values calculated from group means. Bars sharing letters are not significantly different as determined by one-way ANOVA followed by Tukey post-hoc test at $p < 0.05$.

Table 2.3 Relationship between physiological and biochemical responses, and tissue Cd concentration (for measures made in viscera, foot or remaining tissues) or Cd exposure concentration (physiological and haemolymph measures).

Biomarker	Tissue	Coefficient of determination (R^2)	Equation for the line best fit	p - value
Oxygen consumption		0.659	y = -0.2x + 5.1	< 0.01
Ammonia excretion		0.576	y = 0.06x + 2.7	< 0.01
Catalase	Viscera	0.584	y = 0.35x + 29.9	< 0.01
	Foot muscle	0.097	y = 0.08x + 28.8	0.16
	Remaining tissues	0.140	y = 0.008x + 3.4	0.11
Lipid peroxidation	Viscera	0.071	y = 0.003x + 3.5	0.21
	Foot muscle	0.035	y = 0.005x + 1.3	0.38
	Remaining tissues	0.022	y = 0.003x + 1.7	0.48
Glycogen	Viscera	0.338	y = -0.03x + 33.2	< 0.01
	Foot muscle	0.568	y = -0.10x + 14.1	< 0.01
	Remaining tissues	0.109	y = -0.03x + 20.8	0.11
Glucose	Haemolymph	0.865	y = 0.006x + 0.23	< 0.01
Protein	Haemolymph	0.631	y = 0.25x +10.0	< 0.01

Analysis of the effect of Cd exposure on tissue catalase activity showed a significant effect of tissue type ($p < 0.01$), Cd exposure concentration ($p < 0.01$) and also a significant effect of the interaction between these two factors ($p < 0.05$). Post-hoc analysis showed that catalase activity in the viscera of mud snails increased with Cd exposure concentration, and was significantly higher than that in the other tissues. Foot muscle and the remaining tissues did not display a significant induction of catalase activity over the range of concentrations tested (Figure. 2.3A). The increase in catalase response in the viscera correlated strongly with the viscera Cd burden ($R^2 = 0.584$, $p < 0.01$; Table 2.3). A two-way ANOVA highlighted that lipid peroxidation was not significantly impacted by tissue type ($p = 0.07$), Cd exposure concentration ($p = 0.09$) or interaction between these two factors ($p = 0.27$) (Figure 2.3B).

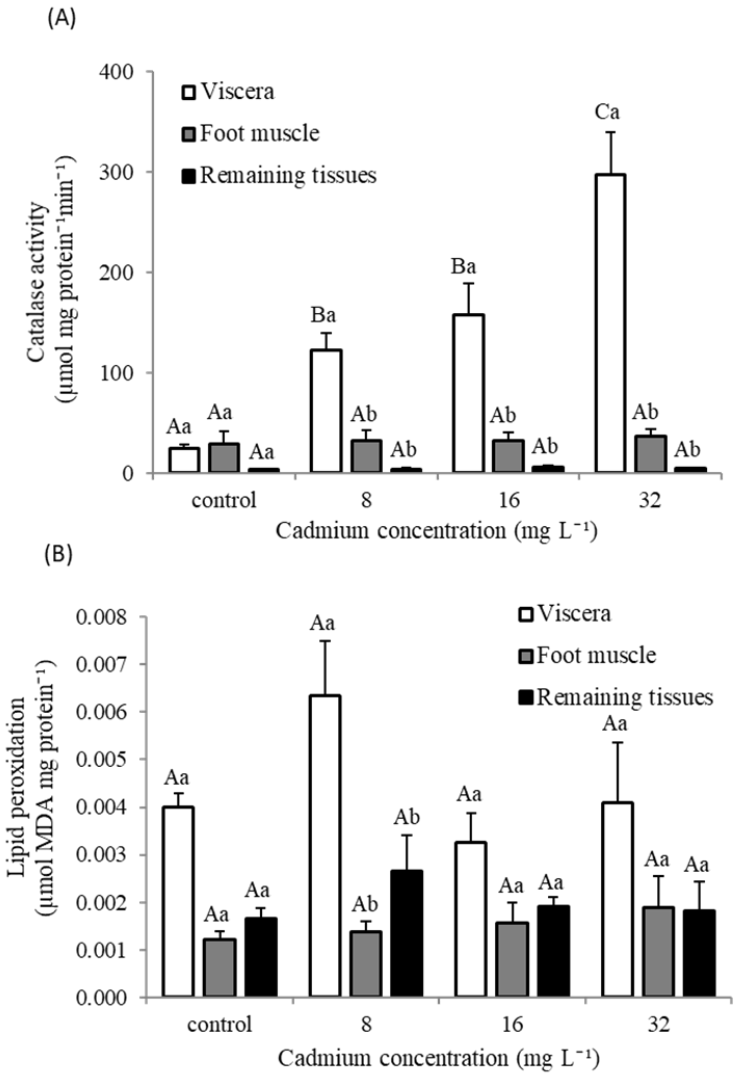


Figure 2.3 The effect of acute (48 h) waterborne Cd exposure on catalase activity (A) and lipid peroxidation (B) in viscera, foot muscle, and remaining tissues in *A. crenata*. Plotted values represent mean ± SEM of 6 replicates. Bars sharing lowercase letters are not significantly different with respect to tissue type within an exposure concentration, while bars sharing uppercase letters are not significantly different between different exposure concentrations, within the same tissue, as determined by two-way ANOVA followed by Tukey post-hoc test at $p < 0.05$.

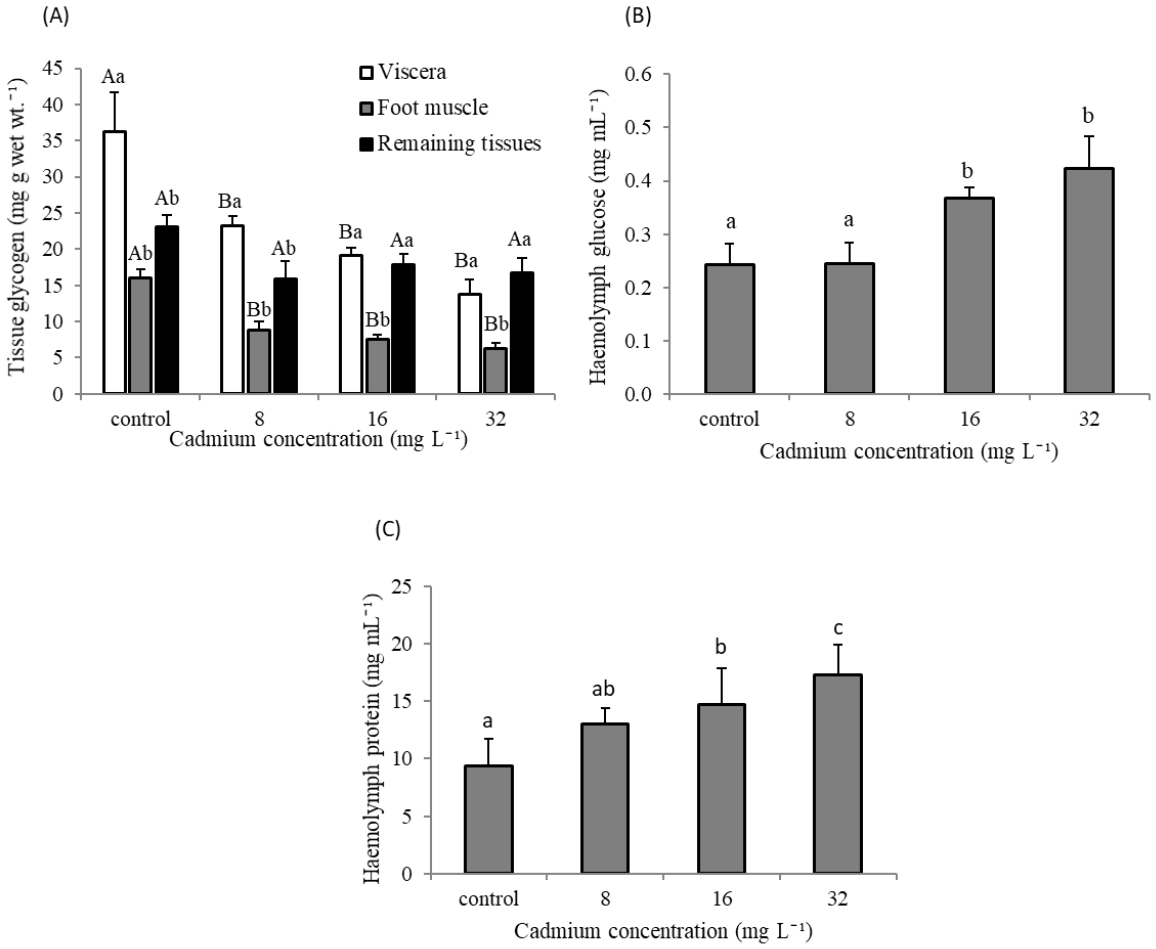


Figure 2.4 The effect of an acute (48 h) Cd exposure on tissue glycogen (A); haemolymph glucose (B); and haemolymph protein (C) in *A. crenata*. Plotted values represent mean ± SEM of 6 replicates. In Panel A, bars sharing lowercase letters are not significantly different with respect to tissue type within an exposure concentration, while bars sharing uppercase letters are not significantly different between different exposure concentrations, within the same tissue, as determined by two-way ANOVA followed by Tukey HSD post-hoc test at $p < 0.05$. In Panels B and C bars sharing letters are not significantly different as determined by one-way ANOVA, followed by Tukey post-hoc test at $p < 0.05$.

Acute Cd exposure significantly altered tissue glycogen levels in *A. crenata.* A significant effect of tissue type ($p < 0.01$) and Cd exposure concentration ($p < 0.01$) were noted, but no significant effect of the interaction between these two factors ($p = 0.11$) was seen. In both viscera and foot muscle, tissue glycogen significantly declined at 8 mg Cd L^{-1}, relative to control values, although a further increase in Cd exposure concentration did not result in further significant reduction (Figure 2.4A). The reduction was 36%, 47% and 62% in the viscera, while foot muscle glycogen declined by 45%, 53% and 61% for 8,16 and 32 mg Cd L^{-1} treatments, respectively. The glycogen levels in the remaining tissues were statistically similar to the control group for all Cd exposures. The trend in viscera ($R^2 = 0.338$, $p < 0.01$) and foot muscle ($R^2 = 0.568$, $p < 0.01$) glycogen concentrations correlated significantly with Cd tissue burdens in these tissues (Table 2. 3).

Relative to control animals, no significant changes in haemolymph glucose level were observed in mud snails exposed to 8 mg Cd L^{-1} (Figure 2.4B). In contrast, significantly increased levels of haemolymph glucose (52% and 75% respectively) were found in snails exposed to 16 and 32 mg Cd L^{-1} (one-way ANOVA; $p < 0.05$). The correlation between haemolymph glucose and Cd exposure concentration was strong and significant ($R^2 = 0.865$, $p < 0.01$; Table 2. 3).

Acute Cd exposure resulted in increased haemolymph protein concentrations in mud snails (Figure 2.4C). For example, mud snails exposed to 16 and 32 mg Cd L^{-1} showed 57% and 85% increases in haemolymph protein levels, significantly elevated relative to the control (one-way ANOVA; $p < 0.05$). Overall, the haemolymph protein concentration correlated significantly with Cd exposure concentration ($R^2 = 0.631$, $p < 0.01$; Table 2.3).

2.4 DISCUSSION

2.4.1 Acute lethal toxicity and tissue accumulation

The 48-h LC_{50} of adult *A. crenata* in the current study was 50.4 mg L^{-1}, a value higher than that recorded previously for a gastropod species. For example, Ramakritinan et al. (2012) reported an LC_{50} value of 39.8 mg L^{-1} for the marine snail, *Cerithedia cingulata*, exposed to Cd at a higher temperature (29 versus 15 °C) and salinity (33 versus 20‰) than that used in the current study. Similarly, *A. crenata* is considerably more tolerant than freshwater pulmonate snails, with a 96-h LC_{50} of 0.35 mg Cd L^{-1} having been reported for *Lymnaea stagnalis* (Pais, 2012).

Many factors are known affect the short-term survival of gastropods to Cd exposure, but salinity is clearly a key determinant. Generally, marine species are more tolerant of Cd than freshwater species (Wang et al., 2010). In seawater, high concentrations of ions such as calcium and chloride can reduce Cd bioavailability by processes of competition and complexation, respectively. Waterborne Cd is only considered to be bioavailable in its charged divalent form (Cd^{2+}), where it gains access to the sensitive internal tissues via dedicated divalent metal ion transporters (e.g. DMT-1), or via calcium uptake pathways, by virtue of its mimicry of divalent calcium (Komjarova and Bury, 2014). Thus, the chemistry of seawater acts to reduce Cd toxicity by the formation of Cd chloride species, or through calcium competition for uptake. There is some evidence to support the suggestion that reduced Cd bioavailability is a factor explaining the differences between *A. crenata* and freshwater pulmonate snails. Pais (2012) reported soft tissue Cd concentrations of ~800 µg g dry wt^{-1} at an exposure concentration of 600 µg L^{-1} in the freshwater snail, and although that study examined bioaccumulation after 96 h, these values are still higher (by around 1.4 to 2-fold) than those in the current study after 48-h exposure to Cd concentrations as high as 32 mg L^{-1} (Figure 2.1). However, it is unlikely that water chemistry alone explains differences in sensitivity between estuarine and freshwater snails. For example, when *A. crenata* sensitivity was calculated based on predicted Cd^{2+} concentration, an LC_{50} value of 3.33 mg L^{-1} was determined. While this is considerably lower than the value based on dissolved Cd, it is still an order an order of magnitude greater than that determined for freshwater pulmonates. Consequently, it is likely that species-specific differences may also be involved in the high tolerance of *A. crenata* to waterborne Cd.

In aquatic macrofauna the gills are particularly vulnerable to waterborne toxicants. To a significant extent the vulnerability of this tissue stems from its multifunctional roles, as exposure of the gill to a metal toxicant can disrupt ion transport, waste excretion and respiratory function (McGeer et al., 2012). *Amphibola* lacks a gill, and instead utilises a lung which functions only in respiration, and which is relatively isolated from the external medium. Although such an explanation does not seem to hold for freshwater pulmonates, it is possible that at least a component of the high tolerance of *A. crenata* may relate to the absence of a gill (Bennington, 1979). In addition, during the Cd exposure, it was noted that many *A. crenata* individuals produced mucus. This layer may act as a protective barrier, binding Cd, and reducing exposure through subsequent sloughing (Betzer and Pilson, 1974). Moreover, the presence of an operculum in gastropods may also promote tolerance. Under unfavourable conditions the operculum may close, limiting exposure of soft tissues to waterborne toxicants such as Cd (Bennington, 1979). This behavior was unable to be verified in the current study.

The results of the present study show that the gonad and digestive gland (grouped together in the current study as "viscera") accumulated the highest tissue burden of Cd in *A. crenata* exposed to waterborne Cd. Tissue burden in this compartment and the remaining tissues (i.e. kidney, mantle, remaining digestive tissues and heart) showed saturation with increasing Cd exposure concentration (Table 2.2). The digestive gland and the kidneys of gastropod molluscs are well recognised as tissues of Cd storage (Nott et al., 1993). These tissues display a number of mechanisms that facilitate a storage role. For example, Cd exposure induces expression of the thiol-rich protein metallothionein in gastropod digestive gland and kidney, which is able to chelate Cd in a biologically inert form (Bebianno and Langston, 1998). In the digestive gland, Cd will associate with phosphate granules, which are also considered to detoxify Cd (Nott et al., 1993).

2.4.2 Energetic responses

In the current study, biochemical and physiological endpoints were measured in *Amphibola* individuals that survived a 48-h exposure to Cd. This does not, however, mean that changes in these endpoints are necessarily representative of mechanisms that facilitate tolerance. In order to determine whether changes in biochemical and physiological responses contributed towards survival, the ultimate fate of those individuals would have to be determined. Measured effects

could, for example, be changes that precede eventual mortality and thus do not benefit the animal. Nevertheless, the responses measured do reflect the effects of Cd exposure, and thus are likely to be informative of pathways by which this important contaminant exerts toxicity. Further research examining responses at more environmentally realistic exposure concentrations will be required to determine the functional importance of Cd-induced changes in parameters such as bioenergetics.

Pulmonate snails are capable of bimodal respiration, and when submerged, perform gas exchange across the cutaneous surface. *Amphibola crenata* displays equivalent rates of oxygen consumption in air and water (Shumway, 1981), suggesting that the determination of respiratory rates and bioenergetics in an aquatic setting provides a valid assessment of the impact of Cd on these endpoints. In the present study, the oxygen consumption rate of *A. crenata* decreased with increasing Cd exposure concentration. Previous studies have explained declines in oxygen consumption upon metal exposures as being a function of damage to the respiratory epithelium, or a protective mucus production response, both of which would inhibit oxygen uptake (Wu and Chen, 2004). It is also important to note that in bivalve molluscs, shell closure in response to Cd exposure has been offered as an explanation for a decrease in feeding activity (Chandurvelan et al., 2012), and if *A. crenata* closed their opercula in response to Cd, preventing access of the lung to the oxygenated medium, then this would also decrease oxygen consumption.

Changes in oxygen transport entities and oxygen demand could also explain the decline in oxygen consumption with waterborne Cd exposure in *A. crenata*. For example, in a study on the effects of Cd in the estuarine crab, *Chasmagnathus granulata,* it was suggested that Cd may alter polymerisation of haemocyanin, thereby reducing haemocyanin oxygen affinity (Rodriguez et al., 2001). Effects on oxygen demand have also been observed, with a significant decline in respiration observed in Cd-exposed mitochondria isolated from a bivalve mollusc, likely due to effects of the metal on mitochondrial function (Sokolova, 2004). All of these factors may have some utility for explaining the observed respiratory impairment in *A. crenata* in the current study, but identifying the specific mechanism(s) of effect requires further research.

Ammonia is the final product of protein catabolism in most aquatic animals (Mayzaud and Conover, 1988). The increase of ammonia excretion in *A. crenata* exposed to Cd (Figure 2.2B), is indicative of an increase in the catabolism of amino acids or other nitrogenous compounds, and is

consistent with previous findings in Cd-exposed molluscs (e.g. Chandurvelan et al., 2012). A stimulation in protein catabolism could reflect either enhanced degradation of proteins damaged by Cd exposure (e.g. Tamas et al., 2014), or a change in energy metabolism as a result of Cd exposure. This latter hypothesis is supported by the significant increase in haemolymph protein in Cd-exposed *A. crenata* (Figure 2.4C). This suggests that protein is being mobilised for use as an energy substrate in this species, and is consistent with similar findings in Cd-exposed bivalve molluscs (Chandurvelan et al, 2013; Spann et al., 2011). Further support for the hypothesis that there is a switch to protein metabolism in Cd-exposed mud snails, is provided by changes in the O:N ratio. Hypothetically, O:N ratios less than 16 represent a protein-dominated catabolism, while values higher than 24 represent the predominant use of lipids and carbohydrates as energy substrates (Mayzaud and Conover, 1988). In control animals in the current study, the O:N ratio of 50 was therefore reflective of a reliance on lipids and/or carbohydrates as fuels. However, the O:N ratio values for *A. crenata* exposed to Cd were less than 16, thus indicating an increased reliance on protein catabolism. Widdows (1978) suggested that an O:N ratio of 7 or less would be reflective of stress condition. This was the case for *A. crenata* exposed to waterborne Cd concentrations of 16 and 32 mg L^{-1}, and similar results have been found for juvenile prawns (*Exopalaemon carinicauda*; Zhang et al., 2014) and green-lipped mussels (*Perna canaliculus*: Chandurvelan et al., 2012), exposed to Cd.

2.4.3 Biochemical endpoints

Catalase is an antioxidant defence enzyme responsible for the reduction of H_2O_2 to water and oxygen (Lushchak, 2011). As H_2O_2 is the main source of the hydroxyl radical, the most reactive and toxic reactive oxygen species (ROS) in living cells, catalase is considered an important biomarker of oxidative stress, and has been extensively investigated in sentinel species, such as mussels, exposed to Cd (Chandurvelan et al., 2013; Koutsogiannaki et al., 2015). The present study showed that catalase activity was induced in viscera of mud snails exposed to waterborne Cd (Figure 2.3A). This suggests that in this tissue Cd perturbed ROS metabolism, resulting in a stimulation of catalase activity. This is an effect that has been seen previously in digestive (Chandurvelan et al., 2013) and reproductive (Peng et al., 2015) tissues (i.e. those tissues which constitute the viscera of *Amphibola*) upon Cd exposure in other marine invertebrates. In contrast, no significant change in catalase activity was found in foot muscle and remaining tissues. This

likely reflects the higher Cd accumulation in viscera relative to the other tissues, a finding supported by the positive correlation between Cd burden and catalase activity in this tissue (Table 2.3).

Impairment of the delicate balance that exists between ROS production, and mechanisms that exist to detoxify ROS, can result in oxidative damage, such as lipid peroxidation (Lushchak, 2011). The lack of any significant Cd-induced lipid peroxidation in the current study (Figure 2.3B) could be explained a number of ways. One possibility is that Cd did not initiate an imbalance in the relationship between ROS production and ROS scavenging. Alternatively, the enhanced activity of antioxidant defences, such as that seen in the viscera for catalase, were sufficient to prevent damage. Similar findings have been observed in Cd-exposed bivalve molluscs (Viarengo et al., 1990; Chandurvelan et al., 2013). It is also possible that lipid peroxidation was not induced over the relatively short time-frame of the current study (48-h), and that a longer exposure period, or a post-exposure sampling point may have provided a different outcome.

Gastropod snails store large quantities of glycogen in their tissues, likely as an energy reserve (Bennington, 1979; Ansaldo et al., 2006). Changes in tissue glycogen can therefore be used as an endpoint that reflects the energy demand of an organism under stress conditions. In the present study, visceral and foot muscle glycogen levels dropped significantly in all Cd treatments. This could be attributed to the utilisation of glycogen reserves by the organism through glycogenesis in order to meet energy demand during stress. Depletion of glycogen in aquatic organisms due to waterborne Cd toxicity has been reported in a number of studies (Leung and Furness, 2001; Ansaldo et al., 2006; Chandurvelan et al., 2013). It is, however, worth noting that all experimental animals had been fasted for 96 h by the time of tissue sampling. It is therefore possible that the responses of tissue glycogen observed in the presence of Cd could have been exacerbated by the absence of external nutrient sources.

Haemolymph glucose concentrations in aquatic organisms have been recognised as a potential biomarker of a variety of anthropogenic stressors, including trace metals (Hall and van Ham, 1998; Lorenzon, 2005). Increases in glucose haemolymph, such as those observed following Cd exposure in the current study, are likely to be derived from stored glycogen (Cameselle et al., 1980), and

this would be consistent with the finding of a reduction in tissue glycogen in *A. crenata*. However, given that physiological data suggested an increased reliance on protein metabolism in Cd-exposed *A. crenata*, it would seem likely that at least a component of the increase in haemolymph glucose was through the conversion of glucogenic amino acids to glucose, thereby accounting for the rise in ammonia excretion rate. This would also be consistent with the observed increase in haemolymph protein induced by Cd in *A. crenata* (Figure 2.4C). Lorenzon et al. (2000) found similar changes in haemolymph glucose to those described here for Cd-exposed mud snails, in the shrimp *Palaemon elegans* treated with sublethal concentrations of Cd for 24 h. Moreover, Jiang et al. (2013) found hyperglycaemic responses in freshwater crayfish, *Cherax quadricarinatus* for up to 96 h of Cd exposure. However, hyperglycaemia, has not been reported in all studies. For example, Bislimi et al. (2013) found a hypoglycaemia in the garden snail, *Helix pomatia*, exposed to industrial pollution. The effects of anthropogenic stressors on haemolymph glucose may, therefore, vary depending on the species.

2.5 CONCLUSIONS

In general, estuarine gastropods are tolerant of environmental extremes, including fluctuations in salinity, dissolved oxygen, temperature, osmolality, and the availability of water (Shumway and Marsden, 1982). The current study show that this high tolerance extends to withstanding the lethal impacts of the trace metal Cd, at least over acute (48-h) exposures, in the estuarine pulmonate mud snail *Amphibola crenata*. This high tolerance contrasts distinctly with the high sensitivity of freshwater pulmonates to Cd (e.g. Das and Khangarot, 2010), even when accounting for water chemistry and the resulting changes in Cd speciation. This suggests that low tolerance to Cd is not solely a function of being a pulmonate snail, but relies on specific physiological characteristics of the different species. This is supported by bioaccumulation data, which show a much greater relative capacity for metal bioaccumulation in the freshwater pulmonates (e.g. Pais, 2012), than in *A. crenata*. In the current study, even though Cd exposure concentrations were significantly in excess of those likely to be found in natural settings, and Cd increased significantly in snail tissues, there was no increase in oxidative stress over the 48-h exposure, likely due to the stimulated activity of antioxidant pathways such as catalase. There were, however, significant changes in energy metabolism, suggesting that chronic Cd exposures would have a more deleterious effect on

mud snail health. The study also recognised that in the environment, Cd uptake can occur through many routes including through diet and sediment (McGeer et al., 2012), which could have distinct effects on bioaccumulation patterns and toxicity.

Chapter 3

Physiological and biochemical responses of the estuarine pulmonate mud snail, *Amphibola crenata*, chronically exposed to waterborne cadmium

3.1 INTRODUCTION

Gastropods are known to concentrate trace metals in their tissues according to the availability of those metals in the environment and thus are considered suitable species to investigate as model species for metal bioindicators. A number of studies have shown that gastropods are sensitive to Cd and accumulate the Cd in body tissues, storing it in a detoxified form (Laskowsk and Hopkin, 1996; Chabicovsky et al., 2004; Chandran et al., 2005; Cunha et al., 2007); However, not all gastropods are sensitive to Cd toxicity in the same manner. The susceptibility of pulmonate gastropods to Cd toxicity strongly depends on their origin, exposure concentration and duration of exposure. The freshwater pulmonate, *Lymnaea stagnalis* showed inhibition of growth at 32 µg Cd L^{-1} exposed for 12 days (Das and Khangarot, 2010), while programmed cell death in the hepatopancreas of the terrestrial pulmonate, *Helix pomatia* occurred when exposed to 0.21 – 0.25 µg Cd L^{-1} for 10 days (Chabicovsky et al., 2004).

The previous study (Chapter 2) on the estuarine pulmonate *Amphibola crenata* has shown that this species is highly tolerant of toxic impacts of the Cd over an acute (48-h) time-frame; However, no studies have been done to assess tolerance and kinetics of Cd bioaccumulation in these estuarine pulmonates under a chronic Cd exposure time-frame. The main objectives of the present study were to investigate sublethal endpoints (i.e. energetic and biochemical responses) in the estuarine pulmonate *A. crenata* to chronically exposed to Cd (21-days). The present study used oxygen consumption, ammonium excretion, O:N ratio (energetic endpoints), catalase activity, lipid peroxidation, glycogen and haemolymph glucose and protein (biochemical endpoints) responses in *A. crenata* as endpoints to chronic Cd toxicity. The study further determined Cd bioaccumulation in different tissues as a function of both exposure concentration and time.

3.2 METHODS

3.2.1 Collection and maintenance of mud snails

Adult mud snails (>18 mm) were collected during December 2015 from the mouth of the Avon-Heathcote Estuary/Ihutai (S 43˚33.125', W 172˚44.775') in Canterbury, New Zealand. The sediment Cd concentration near the site of collection was 0.1 µg g^{-1} (Chandurvelan et al., 2016).

The selection of adult mud snail for the experiment was attributed to easy handling and transportation, ability of individual mud snail to provide measurable physiological responses within a reasonable time frame and to provide enough tissues for biochemical assays, compared to small and medium-sized mud snails. Furthermore, snail sample collection was conducted during the summer owing to high abundance of snails. Prior to the experiment, the snails were acclimatized for 48 hours under laboratory conditions (15±0.5 °C with 12 hours light :12 hours dark) in a holding tank containing 2 L of 20 ppt filtered natural seawater prepared as described in Chapter 2. During acclimation, snails were not fed to preclude any unwanted variations in their physiology prior to the experiment.

3.2.2 Experimental protocol

The chronic (21 days) Cd exposure test was carried out in acid-washed 1.5 L polypropylene containers at 15°C and 20 ppt salinity (natural SW as described in Chapter 1) at four nominal Cd concentrations (0, 0.2, 4 and 8 mg Cd L^{-1}). Day/night cycles (12 hours light: 12 hours dark) were maintained throughout the acclimation and experiment period. The highest concentration of Cd (8 mg L^{-1}) chosen was similar to the lowest concentration of the acute (48 hours) Cd exposure (Chapter 2), where no mortality occurred during the acute time-frame and the lower value (0.2 mg L^{-1}) was chosen to represent a high environmental exposure scenario (Chandurvelan et al., 2012). All containers were aerated throughout the experiment. A cadmium chloride stock solution containing 1 g L^{-1} Cd (as $CdCl_2.2½\ H_2O$) was used to achieve the concentrations used in the experiment. For all exposure concentrations, six replicates were used, with 15 individual mud snails per replicate. Snails were assigned randomly to the different treatments. Snail mortality was monitored at 48 h intervals, and snails were not fed during the exposure. Mortality was assessed when immobile mud snail failed to respond to probing using forceps and if found was removed from the system. Seawater was changed every 48 hours during the experiment. Water samples (15 mL at each time) from all treatments were collected at the beginning of the experiment, then before and after each water change until the end of the experiment, by filtering through a Millex 0.45 µm filter (Millipore Ltd, Cork, Ireland). Water samples were subsequently acidified to pH < 2 using 70% ultrapure HNO_3 and stored at 4°C until analysed by Atomic Absorption Spectroscopy as described in Chapter 2 (Section 2.2.4). In order to determine exposure concentration, at each replicate, initial and final Cd concentrations within each water change were averaged and these

values were averaged across all water change within the exposure. This mean value was then averaged across all replicates.

3.2.3 Bioaccumulation and sublethal endpoints

Two groups of snails were assessed for physiological responses (i.e. oxygen consumption, ammonia excretion and O:N ratio; n = 6), while a separate group of animals was assessed for tissue Cd bioaccumulation (n = 6) and for biochemical assays (catalase activity, lipid peroxidation, glycogen, haemolymph glucose and protein; n=6) on Days 7,14, and 21 of exposure. During the experimental period, 65% and 100% mortality occurred in 4 and 8 mg Cd L^{-1} exposures respectively. At Day 14, these were therefore excluded from endpoint determinations for Days 14 and 21. Sublethal endpoints for these concentrations were found only for Day 7. Detailed protocols for all of the tissue bioaccumulation, physiological and biochemical measurements are provided in Chapter 2 (sections 2.2.3 & 2.2.4). In order to estimate O:N ratio, oxygen consumption and ammonia excretion were measured in separate animals. Therefore, only mean values were used.

3.3 RESULTS

The nominal and measured concentrations of Cd for each exposure treatment are presented in Table 3.1. The measured Cd concentrations were correlated within nominal Cd values with little variation among replicates.

Table 3.1 Nominal and measured concentration (mg L^{-1}) of Cd analysed in 20 ppt seawater from chronic exposures. Measured values represent the mean exposure level (average of initial and final concentrations measured at each water change). Values are expressed as mean ± SD (n = 2-5).

Concentrations	
Nominal mg L^{-1}	Measured mg L^{-1}
0	0.021 ± 0.003
0.2	0.28 ± 0.002
4	4.36 ± 0.05
8	8.59 ± 0.06

3.3.1 Mortality

The percent mortality of *A. crenata* chronically exposed to cadmium over 21 days are shown in Fig. 3.1. No mortality occurred in control animals throughout the experiment. As concentrations of cadmium increased there was increased snail mortality. The first snail mortalities were recorded at 8 mg L^{-1} after 4 days, with 100% mortality observed after Day 12; whereas 100% mortality rates were recorded after Day 17 in 4 mg L^{-1}. In comparison, average mortality of 3% occurred after Day 16 in the 0.2 mg Cd L^{-1} exposure.

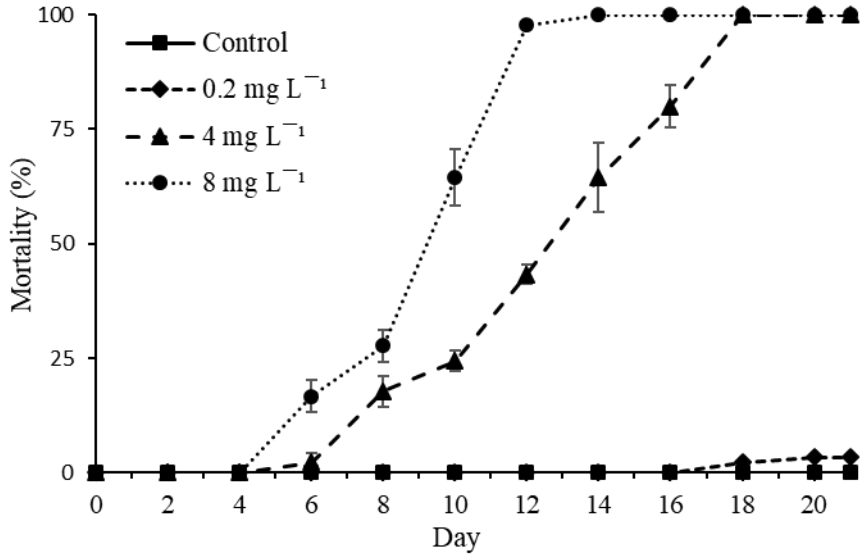


Figure 3.1 Mortality in *Amphibola crenata* following chronic exposure to Cd.

3.3.2 Cd bioaccumulation

The Cd concentration of the control mud snail group remained below 10.5 µg g dry $wt.^{-1}$ over the 21 days exposure. Increasing waterborne Cd concentration and exposure time led to increasing metal accumulation in all tissues (Table 3.2). Cd concentration in all tissues (viscera, foot muscle, and remaining tissues) of mud snails exposed to chronic Cd showed a significant effect of treatment ($p < 0.01$), time ($p < 0.01$), and also a significant interaction ($p < 0.01$) between these two factors. In all Cd exposure concentrations and over the course of exposure time the viscera (gonad and digestive gland) contained significantly higher Cd concentrations, than those found in

the foot muscle and remaining tissues (i.e. kidney, mantle, remaining digestive tissues, and heart). For all tissues, at each sampling time (i.e. day 7, 14, and 21), all Cd exposure concentrations resulted in tissue accumulation levels that were significantly higher than those of unexposed control.

Table 3.2 Bioaccumulation of Cd in different tissues of *Amphibola crenata*. Values are expressed as mean ± SEM (n = 5 – 6). n/a – not available.

Tissue	Duration of Exposure (days)	0 (mg Cd L^{-1})	0.2 (mg Cd L^{-1})	4 (mg Cd L^{-1})	8 (mg Cd L^{-1})
Viscera	7	9.8 ± 1	151 ± 25	3531 ± 933	3959 ± 803
	14	10.1 ± 0.9	656 ± 61	n/a	n/a
	21	7.6 ± 1.1	1649 ± 216	n/a	n/a
Foot muscle	7	2.9 ± 0.4	47.3 ± 5.9	693 ± 94	1249 ± 89
	14	4.6 ± 0.6	99.9 ± 4.3	n/a	n/a
	21	0.9 ± 0.05	149 ± 7	n/a	n/a
Remaining Tissues	7	5 ± 1.1	51.2 ± 5.5	959 ± 92	1867 ± 113
	14	3.2 ± 0.8	81.7 ± 3.8	n/a	n/a
	21	1.2 ± 0.2	105 ± 11	n/a	n/a

3.3.3 Energetic responses

The oxygen consumption (average of 6.9±1 μmol O_2 g^{-1} h^{-1}) of the control mud snail group did not show any significant changes over the period of chronic Cd exposure; However, significant differences from the control were recorded in Cd-exposed mud snail groups (Fig. 3.2A). The effect of Cd exposure on *A. crenata* oxygen consumption was significantly altered by treatment (two-way ANOVA; post-hoc; $p < 0.01$), and time ($p < 0.01$), but there was no significant effect of the interaction between these two factors ($p = 0.15$). Compared to control group, snails exposed to 0.2 mg Cd L^{-1} had similar oxygen consumption on Day 7($p = 0.24$), but significantly reduced ($p < 0.01$) on Day 14 and the effect persisted until Day 21.

The ammonia excretion rates (average of 5.2 ± 0.1 NH_3 g^{-1} h^{-1}) of the control mud snails remained unchange over the course of chronic Cd exposure; However, for Cd-exposed mud snails, excretion rate was significantly affected by Cd concentration (two-way ANOVA; post-hoc; $p < 0.01$) and time ($p < 0.05$) and also exhibited an interaction between these two factors ($p < 0.05$) (Fig. 3.2B).

The snails exposed to 0.2 mg Cd L^{-1} displayed no significant changes ($p = 0.13$) for ammonia excretion on Day 7, while a significant increase ($p < 0.01$) in ammonia excretion was observed on Days 14 and 21. The O:N ratio of *A. crenata* showed that molar ratio of oxygen consumed to ammonia excreted declined with both increasing Cd exposure concentration and duration of exposure.

On Day 7, O:N in control conditions was 48, while the O:N ratio values of 46, 12, and 13 were recorded at Cd exposure concentrations of 0.2, 4 and 8 mg Cd L^{-1}, respectively (Fig. 3.2C). Values of 5 and 3 were found at Cd exposure concentration of 0.2 mg Cd L^{-1} for Days 14 and 21 respectively, compared with control values of 27 and 36; However, O:N ratios were based on group mean of oxygen consumption and ammonia excretion derived from separate groups of snails, thus were not able to be compared statistically (see section 3.2.3).

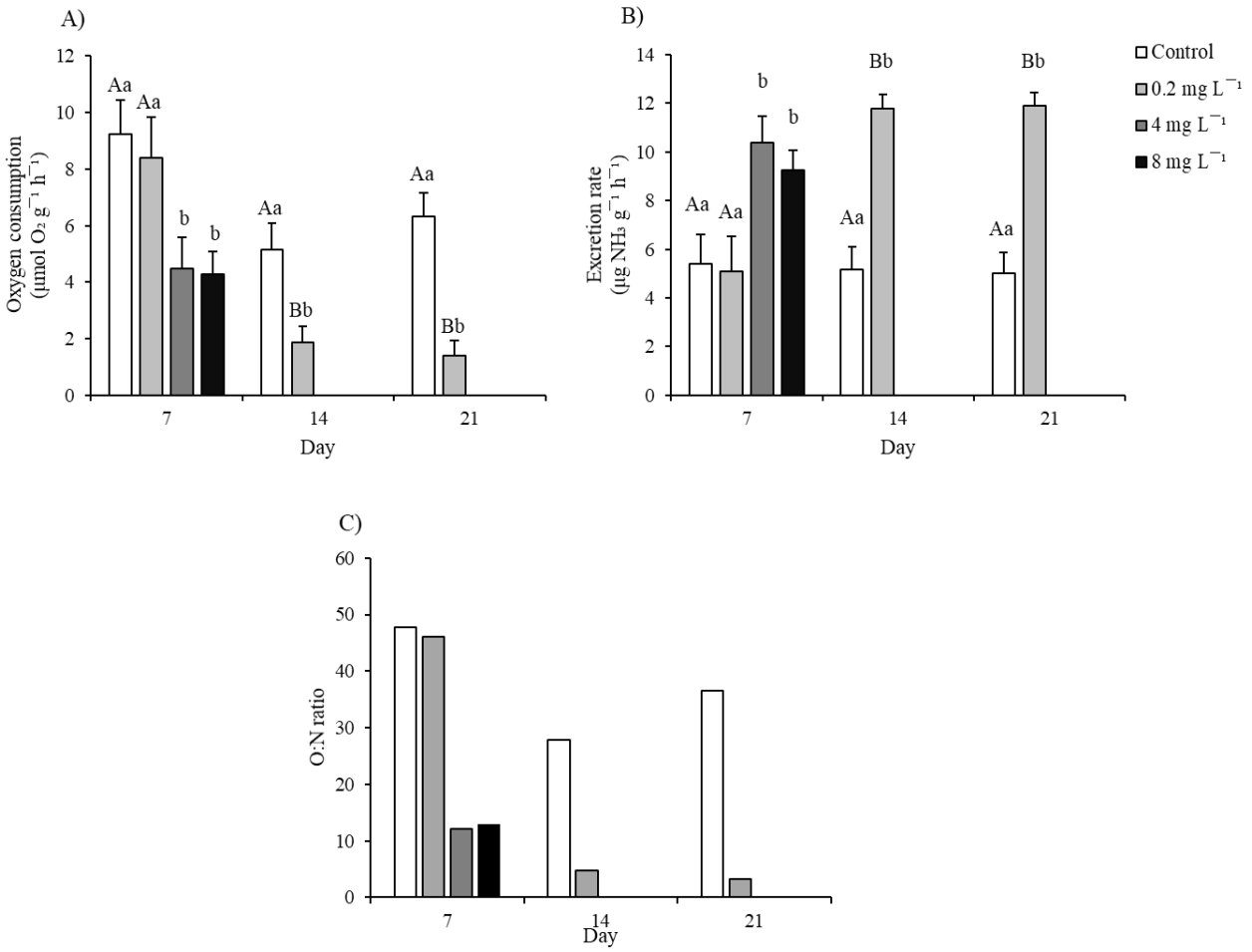


Figure 3.2 The effect of a chronic (21 days) Cd exposure on oxygen consumption (A); ammonia excretion (B); and O:N ratio (C) in *A. crenata*. Plotted values of fig. (A) and (B) represent mean ± SEM of 6 individuals, while values of fig. (C) represent O:N ratio that obtained from oxygen consumption and ammonia excretion results. Fig. (A) and (B) data were analysed by two-way ANOVA, followed by Tukey HSD post-hoc test at $p < 0.05$ with exposure concentration and time as factors. Bars sharing lowercase letters are not significantly different with respect to exposure concentration within a day of sample, while bars sharing uppercase letters are not significantly different with respect to exposure time within an exposure concentration.

3.3.4 Biochemical endpoints

The catalase activity of mud snails was significantly affected by Cd concentration. Catalase activity in the viscera of Cd-exposed mud snails was significantly higher than that in the other tissues throughout the 21 days of chronic Cd exposure (Fig 3.3A). For the viscera, there were no significant interaction effects between the two factors (two-way ANOVA; post-hoc; $p = 0.96$) although significant effects of exposure concentration ($p < 0.01$) and of time ($p < 0.05$) were observed. Moreover, catalase activity in the viscera was increased in the 0.2 mg L^{-1} Cd-exposed snails throughout the 21 days of chronic exposure. Catalase activity in the foot muscle and the remaining tissues were significantly affected by treatment as a factor ($p < 0.01$), but there was no significant effect of time ($p = 0.86$ and 0.26, respectively) and interaction between exposure concentration and time ($p = 0.70$ and 0.18, respectively; Fig. 3.3B and 3.3C) were observed. Nevertheless, catalase activity in the remaining tissues was significantly increased in the 0.2 mg L^{-1} Cd-exposed snails on Day 14 relative to time-matched control group, but which had dissipated on Day 21.

Lipid peroxidation levels in Cd-exposed snails showed a significant variation with respect to the treatment and exposure time. Within each sampling time, viscera of Cd-exposed snails showed significantly higher levels of lipid peroxidation compared to the time-matched control snails (Fig. 3.4A). For viscera lipid peroxidation level, there was a significant effect for treatment (two-way ANOVA; post-hoc; $p < 0.01$), and exposure time ($p = 0.04$), but no interaction between these two factors ($p = 0.52$). For the lipid peroxidation levels in the foot muscle (Fig. 3.4B) and the remaining tissues (Fig. 3.4C), there was a significant effect of treatment ($p < 0.01$) and exposure time ($p < 0.01$), and interaction between these two factors in the foot muscle ($p < 0.01$). The lipid peroxidation level in the foot muscle was reduced in the 0.2 mg L^{-1} Cd-exposed snails throughout the 21 days, with a significantly lower level on Day 21 than that of the control snails.Throughout the experiment, the chronic Cd exposure resulted in significantly lower levels of glycogen in all tissues in all Cd treatments compared to the time-matched control group. Glycogen level in the viscera (Fig. 3.5A) was significantly affected by exposure concentration (two-way ANOVA; post-hoc; $p < 0.01$), however, there were no significant effects of exposure duration ($p = 0.09$) nor interaction between these two factors ($p = 0.85$) observed. Conversely, glycogen level in the foot muscle (Fig. 3.5B) was significantly affected by time of exposure ($p < 0.01$), while remaining

tissues (Fig. 3.5C) did not show any significant relationship with both exposure concentration ($p = 0.39$) and time ($p = 0.07$) or to interaction between these two factors ($p = 0.80$).

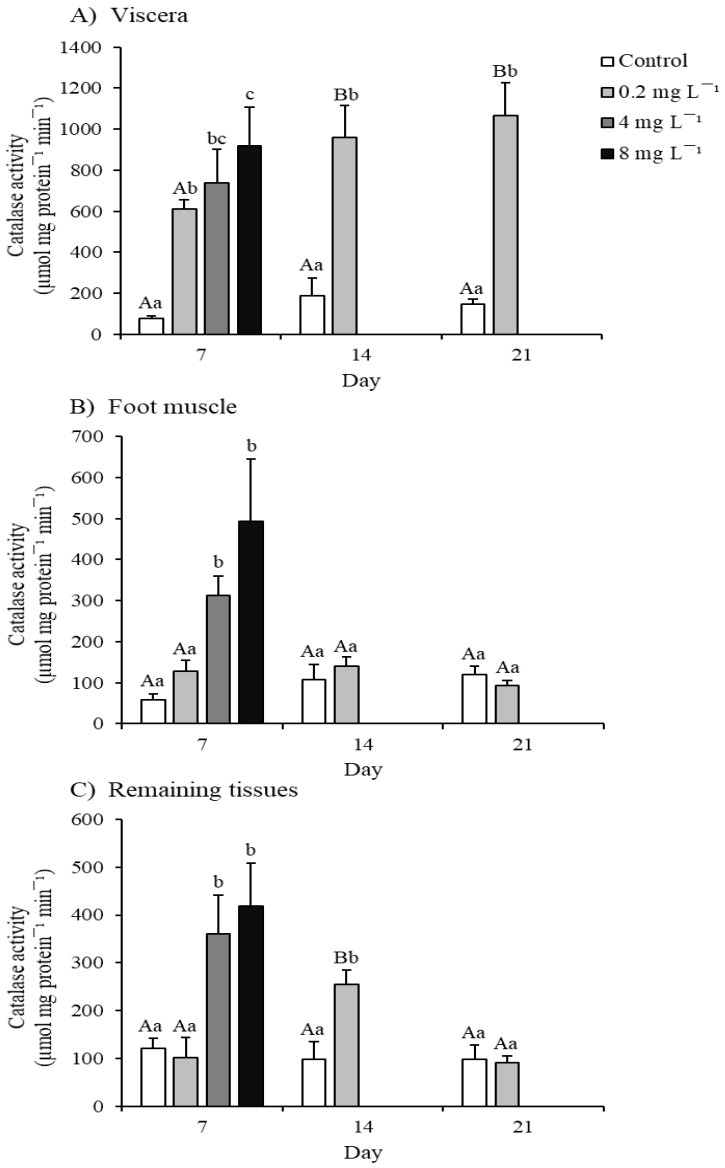


Figure 3.3 Catalase activity (mean ± SEM) in the mud snail (*A. crenata*) viscera (A); foot muscle (B); and remaining tissues (C) after chronic Cd exposure (n = 6; 21 days). Data in Fig. (A) were log-transformed prior to statistical analysis. Bars sharing lowercase letters are not significantly different with respect to exposure concentration within a day of sample, while bars sharing uppercase letters are not significantly different with respect to exposure time within an exposure concentration, as determined by two-way ANOVA followed by Tukey HSD post-hoc test at $p < 0.05$.

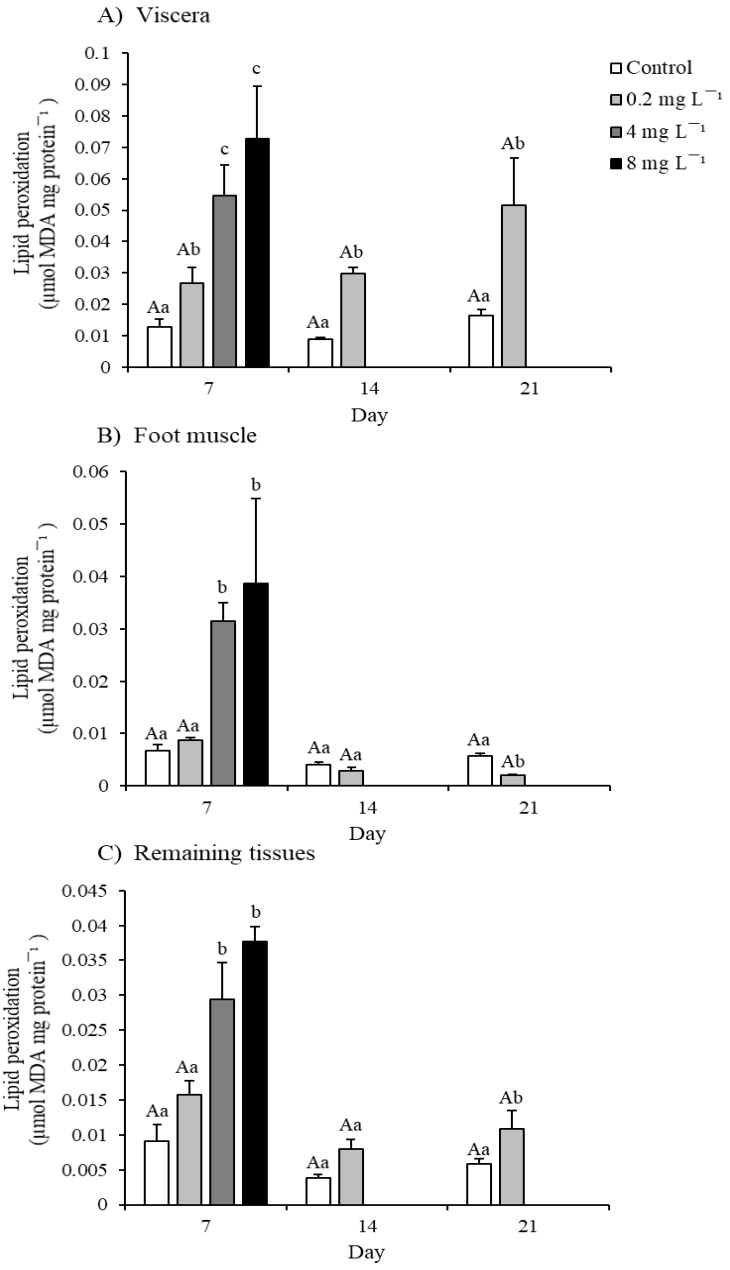


Figure 3.4 Lipid peroxidation level (mean ± SEM) in the mud snail (*A. crenata*) viscera (A); foot muscle (B); and remaining tissues (C) after chronic Cd exposure (n= 6; 21 days). Data in Fig. (A) and (B) were log-transformed prior to statistical analysis. Bars sharing lowercase letters are not significantly different with respect to exposure concentration within a day of sample, while bars sharing uppercase letters are not significantly different with respect to exposure time within an exposure concentration, as determined by two-way ANOVA followed by Tukey HSD post-hoc test at $p < 0.05$.

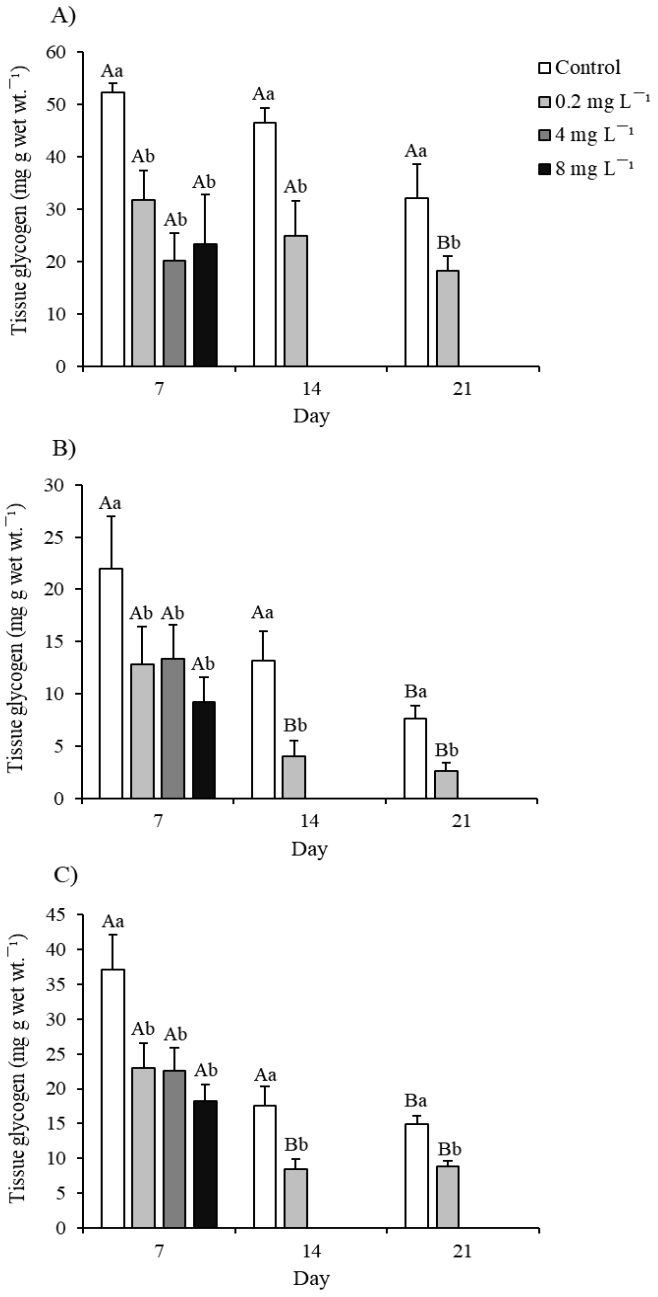


Figure 3.5 Tissue glycogen level (mean ± SEM) in the mud snail (*A. crenata*) viscera (A); foot muscle (B); and remaining tissues (C) after chronic Cd exposure (n= 6; 21 days). All other details are identical to those in Fig.3, except that none of the data were log-transformed. Bars sharing lowercase letters are not significantly different with respect to exposure concentration within a day of sample, while bars sharing uppercase letters are not significantly different with respect to exposure time within an exposure concentration, as determined by two-way ANOVA followed by Tukey HSD post-hoc test at $p < 0.05$.

Relative to time-matched control mud snails, no significant changes in haemolymph glucose levels were observed in mud snails exposed to 0.2 mg Cd L^{-1} on Day 7. In contrast, significantly increased levels of haemolymph glucose (145% and 177% respectively) were measured in snails exposed to 4 and 8 mg Cd L^{-1} on Day 7 (two-way ANOVA; post-hoc; $p < 0.01$; Fig. 3.6A). A significant decrease in haemolymph glucose was observed on Day 14 and 21 in the 0.2 mg L^{-1} Cd-exposed mud snails compared to time-matched control groups (two-way ANOVA; post-hoc; $p < 0.05$). Although there was a significant overall effect of exposure concentration ($p < 0.01$) on haemolymph glucose, the effect of time ($p = 0.52$), and the interaction between exposure concentration and time ($p = 0.09$) were not significant.

Haemolymph protein levels of mud snails exposed to 4 and 8 mg Cd L^{-1} were significantly higher (two-way ANOVA; post-hoc; $p < 0.05$) than those measured in controls after Day 7. In contrast no significant changes in protein were observed in snails exposed to 0.2 mg Cd L^{-1} on Day 7 ($p = 0.98$) and 14 ($p = 1.0$), however, elevated protein levels ($p < 0.05$) relative to unexposed snails on Day 21 (Fig. 3.6B) were observed. Analysis of the overall effect of chronic Cd exposure on haemolymph protein showed a significant effect of exposure concentration ($p < 0.01$), time ($p < 0.01$) and also a significant effect of interaction between these two factors ($p < 0.05$).

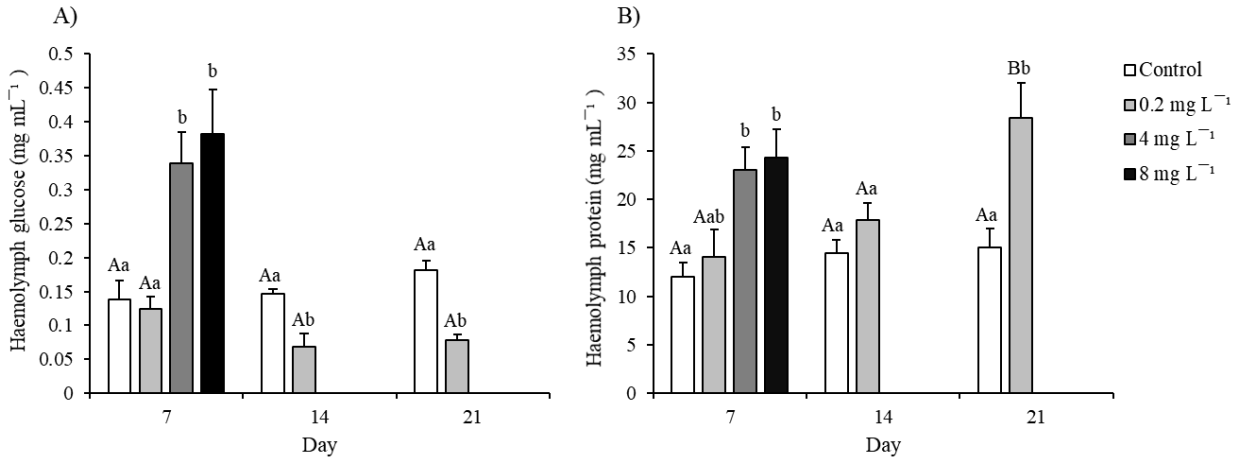


Figure 3.6 The effect of a chronic (21 days) Cd exposure on *A. crenata* haemolymph glucose (A); and haemolymph protein (B). Plotted values represent mean ± SEM of 6 replicates. Data in fig. (A) were log-transformed prior to statistical analysis. Bars sharing lowercase letters are not significantly different with respect to exposure concentration within a day of sample, while bars sharing uppercase letters are not significantly different with respect to exposure time within an exposure concentration, as determined by two-way ANOVA followed by Tukey HSD post-hoc test at $p < 0.05$.

3.4 DISCUSSION

While the chronic effects of cadmium toxicity to freshwater pulmonate snails is well-documented (Coeurdassier et al., 2003, 2004; Reategui-Zirena et al., 2016, 2017), there has been no comparable research for estuarine or marine pulmonate snails. Therefore, in the present study, a long-term Cd toxicity experiment was performed over 21 days. The cadmium compartmentalisation in different tissues (i.e. viscera, foot muscle, and remaining tissues), physiological and biochemical responses in the marine pulmonate snail, *Amphibola crenata* were monitored at specific time intervals (i.e. 7, 14, and 21 Day).

3.4.1 Chronic toxicity and tissue Cd accumulation

Like many other gastropods, accumulation of Cd in tissues of *A. crenata* was both dose- and time-dependent (Brix et al., 2011; Besser et al., 2016). Cadmium burden showed significant variations in the internal fractionations, reflecting differences in the uptake and detoxification mechanisms associated with different tissues. The viscera of this species accumulated higher levels of Cd than foot muscle and remaining tissue (e.g. kidney, mantle, remaining digestive tissues, and heart) at all time intervals and concentrations tested. Similar results have been previously noted in numerous studies of freshwater pulmonate gastropods (Adewunmi et al., 1996; Gundacker, 2000; Coeurdassier et al., 2003; Das and Khangarot, 2010). For example, the viscera of snail, *Lymnaea stagnalis* accumulated 9.7 times more Cd than the foot after exposure to 0.1 mg Cd L^{-1} for 8 weeks (Reategui-Zirena et al., 2017), while the same species exposed to 0.16 mg Cd L^{-1} for 4 weeks reported 2.5 times higher Cd levels (Coeurdassier et al., 2003). Similarly, Das and Khangarot (2010) found 3 times more Cd concentration in the viscera tissue than in the foot when exposed to 0.32 g Cd L^{-1} for 7 weeks. The present study found 11 and 16 times higher levels of Cd in the viscera tissue of *A. crenata* compared to the foot muscle and remaining tissues, respectively, after exposed to 0.2 mg Cd L^{-1} for 3 weeks. These results suggest that the viscera tissue of *A. crenata* has an ability to store metals in high concentrations.

The digestive gland is recognised as a major site of biotransformation of xenobiotic substances in gastropods (Ali et al., 2012; Chandran et al., 2015) and involves a number of mechanisms that may facilitate the transformation/storage role including detoxifying via metal-binding proteins (i.e.

metallothioneins) and incorporation into specific granules (i.e. phosphate granules; Chapter 2). After Day 7 of Cd exposure, snails exposed to 4 and 8 mg Cd L^{-1} concentrations exhibited evidence of Cd saturation in the viscera, while foot muscle and remaining tissues showed a linear relationship with the exposure concentrations (Table 3.3). This suggests that the high Cd exposure exceeded the capacity of viscera to accumulate the metal and generated a 'spill over' of metal. This may have resulted in toxic impacts, which may explain the observed high mortality of snails exposed to 4 and 8 mg Cd L^{-1} after Day 7. In contrast, *A. crenata* exposed to 0.2 mg Cd L^{-1} exhibited linear accumulation of Cd for all tissues over the exposure period, suggesting an ability of these snails to sequester Cd under low Cd exposure concentrations. This outcome is consistent with the effect observed in acute and subchronic exposures of other molluscs such as mussels to waterborne Cd (Erk et al., 2005; Long et al., 2010; Chandurvelan et al., 2012).

Table 3.3 Relationship between concentration of Cd exposure and bioaccumulation of Cd in different tissues of *A. crenata* after Day 7.

Tissue	Coefficient of determination (R^2)	Equation for the line of best fit	p - value
Viscera	0.999	$y = 5208x/(2.2 + x)$	< 0.01
Foot muscle	0.997	$y = 156.4x + 21.1$	< 0.01
Remaining tissues	0.999	$y = 233.3x + 8.9$	< 0.01

3.4.2 Energetic responses

Metabolic processes such oxygen consumption and ammonia excretion of an organism reflect its consumption of energy (Urbina and Glover, 2015). In aquatic organisms, both of these processes are reported to be adversely affected by the presence of trace metals (Barbieri and Paes, 2011; Chandurvelan et al., 2012,2013). Therefore, measurements of oxygen consumed and ammonia excreted by an organism under environmental contamination provide a good indication of energy availability to maintain its vital cellular processes and fulfill the energy demand due to environment and biological alterations. In the present study, the oxygen consumption rate was significantly affected by chronic Cd exposure, therefore suggest potential utility of *A. crenata* oxygen consumption as an indicator for environmental contamination. Although the responses of oxygen consumption to Cd exposure vary greatly according to exposure duration, Cd concentration

and species tested, most literature records are consistent with the findings of the decreased oxygen consumption in *A. crenata* exposed to Cd toxicity. For example, Chandurvelan et al., (2012) reported decreased oxygen consumption in green-lipped mussel, *Perna canaliculus* exposed to Cd for 28 days. Similarly, a significant decrease in oxygen consumption was observed in the mussel *Perna viridis* after 1 week of Cd exposure (Cheung and Cheung, 1995). Sivaramakrishna et al. (1991) found decrease in oxygen consumption by freshwater snail, *Pila globosa* exposed to acute levels of mercury. A number of cellular, physiological and biochemical alterations could explain the decline in oxygen consumption with chronic waterborne Cd exposure in *A. crenata.* For example, damage to the respiratory epithelium, protective mucus production, closes of opercula in response to Cd, changes in oxygen transport entities and oxygen demand, reducing haemocyanin oxygen affinity and effects of the metal on mitochondrial function (Soegianto et al., 1999; Rodriguez et al., 2001; Chapter 2).

In the current study, the ammonia excretion rate of *A. crenata* increased with both increasing Cd exposure concentration and length of exposure, reflecting an increase in the catabolism of proteins as a result of either enhanced degradation of proteins damaged by Cd exposure, or a change in energy metabolism in respect to Cd exposure (e.g. Tamas et al., 2014; Chapter 2). Theoretically, O:N ratios less than 16 represented a protein-dominated catabolism and ratio of 8 or less represented the use of protein as the sole source of energy (Mayzaud and Conover, 1988), while value higher than 24 relate to predominant use of lipids and carbohydrates as an energy source (Mayzaud and Conover, 1988). Following long-term Cd exposure, control animals in the current study, the O:N ratios remained greater than 24 over the period of experiment, reflecting a reliance on lipids and/or carbohydrates as energy sources; However, the O:N ratio values for *A. crenata* exposed to 4 and 8 mg Cd L^{-1} concentrations on Day 7 were less than 16, thus indicating an increased reliance on protein as an energy substrate. The O:N ratio values less than 8 was reported for snails exposed to 0.2 mg Cd L^{-1} on Day 14 and 21, indicating exclusive use of protein as an energy source, suggests that the prolonged starvation during the experimental period and Cd exposure forced the snails to metabolise internal sources of protein as a last resort. This has been also reported previously in green lipped mussels (Chandurvelan et al., 2012). Therefore, the reduced O:N ratios in *A. crenata* found in the present study support the scenario that there is a switch to protein metabolism in Cd-exposed mud snails. In addition, the results of the present study

indicate that Cd has no affect on oxygen consumption, ammonia excretion and O:N ratio of *A. crenata* when they exposed for a relatively low concentration of Cd over a short period of time (i.e. snails exposed to 0.2 mg Cd L^{-1} for 7 days), but significantly altered physiological responses could be observed when the exposure is continued for a relatively longer period (i.e. Day 14 and 21).

3.4.3 Biochemical responses

Catalase is a first line of defense in antioxidant system protecting organisms from oxidative stress (Atli and Grosell, 2016). It reduces H_2O_2, the main source of the hydroxyl radical (OH^-), into water and oxygen to maintain cellular homeostasis. Many studies have found varying responses of catalase activity to increased metal concentrations and to length of metal exposure (Ali et al., 2012: Chandurvelan et al., 2012; Atli and Grosell, 2016). The present study observed a significantly increased catalase activity in viscera of Cd-exposed snails. This likely reflects the higher Cd accumulation in viscera (i.e. digestive gland and gonads) relative to the foot muscle and remaining tissues. The digestive gland of molluscs has been recognised as an important site of trace metal-induced maximal free radical generation (Ali et al., 2012), which could stimulate antioxidant enzymes activities in those tissues.

Cd is a well-known toxic metal, which produces ROS. In general, organisms anti-oxidant defense system removes most of the ROS by increasing its activity in cells (i.e. catalase activity), thus maintaining a delicate balance between ROS production and detoxification. However, overload of Cd in tissues can cause impairment of antioxidant defense mechanisms, resulting oxidative damage, such as lipid peroxidation (Lushchak, 2011). The previous study on *A. crenata* stress responses to acute (48 h) waterborne Cd (Chapter 2) showed a lack of significant Cd-induced lipid peroxidation in snail tissues. In contrast, the present study showed an enhanced lipid peroxidation in snail tissues, indicating that these snails are incapable of maintaining oxidative stress homeostasis when exposed to cadmium for a longer duration. Overall, the present study highlights that the long-term exposure to Cd induced oxidative stress in *A. crenata* by multiple mechanisms including antioxidant defenses and tissue damage.

In gastropod snails, glycogen is considered as a main energy reserve and represents the readily mobilizable storage form of glucose (Leung and Furness, 2001; Ansaldo et al., 2006). Changes in tissue glycogen reflects the energy demand of an organism under stress conditions, therefore, can be used as a useful indicator of stress (Chapter 2). In the present study, long-term waterborne Cd exposure resulted in significant reduction of glycogen in all tissues tested, reflecting the utilization of glycogen reserves by the organism in order to meet energy demand due to stress caused by Cd. Similar results have been reported for freshwater snail, *Biomphalaria glabrata* (Ansaldo et al., 2006). Decreased glycogen levels after cadmium exposure, have also been reported for mussels (Chandurvelan et al., 2013).

Haemolymph metabolites such as glucose and protein have been identified as indicators of physiological condition in organisms exposed to different environmental contamination (Rosas et al., 2004; Rosas et al., 2007). Cd-exposed *A. crenata* exhibited dose-dependent variation in haemolymph glucose as high Cd concentrations (4 and 8 mg Cd L^{-1}) resulted in significantly higher levels of haemolymph glucose (i.e. hyperglycemia). This could be attributed to the utilisation of glycogen reserves by the organism through glucogenesis and/or through the conversion of glucogenic amino acids (Chang, 2005). This first hypothesis is supported by the significant decrease in tissue glycogen in *A. crenata* and the latter hypothesis is corroborated by the increase in ammonia excretion rate and is consistent with the observed increase in haemolymph protein. However, contrary to expectation, haemolymph glucose levels declined significantly in snails exposed to 0.2 mg Cd L^{-1} at Day 14 and 21. This could be attributed to shortage of glycogen derived glucose supply into haemolymph as glycogen reserves of snails significantly reduced with the Cd exposure continued. In contrast, increased levels of haemolymph protein coupled with high ammonia excretion rate in snails exposed to 0.2 mg Cd L^{-1} at Day 14 and 21 supported protein derived energy metabolism by *A. crenata* as a last resort if the Cd-induced stress continued.

3.4.4 Effect of starvation

During the 21 d experiment, snails were not fed. This may have compromised the results because a number of studies have demonstrated that starvation can adversely affect biochemical and physiological responses in aquatic organisms (Vianey-Liaud and Lancastre, 1994; Borges et al.,

2004; Tamburi and Martin, 2016). For example, a study on the gastropod *Magalobulimus oblongus*, Rossi and da Silva (1993) reported decreased levels of glycogen in snail tissues and unchanged glucose levels in haemolymph after 2 weeks of starvation. Reduced levels of glycogen, protein and lipid in foot, gonad-digestive gland complex and viscera were observed in the intertidal gastropod, *Morula granulate* following 70 days of starvation (Devi et al., 1986). Several studies, however, have shown that starvation may not always impact on all biochemical and physiological responses in the same manner. For example, in the golden mussel, *Limnoperna fortune*i starvation over 63 days resulted in no weight loss (Cordeiro et al., 2016). Unchanged oxygen consumption rate was observed in the Northern abalone, *Haliotis kamtschatkana* over 27 days of starvation (Carefoot et al., 1993). In the present study *A. crena*ta had 100% survival and biochemical and physiological responses were unchanged in control mud snail group over the 21 d of the experiment. This suggests that a significant proportion of the observed biomarker responses were related to the Cd toxicity. However, it is worth studying further the impacts of starvation on the biochemistry and physiology of *A. crena*ta using starved and newly collected individuals.

3.5 CONCLUSIONS

In the present study, significant differences in the responses of *A. crenata* to chronic Cd exposure were observed as a function of both exposure concentration and exposure duration. The chronic Cd exposure is likely to be representative of the conditions experienced by this snail in its natural environment. A number of the physiological and biochemical responses investigated in the present study were significantly affected by both Cd exposure concentration and duration. This finding illustrated that these mud snails accumulate Cd in key tissues and show physiological and biochemical impairment in response to chronic Cd exposure. In addition, the results highlighted Cd-induced oxidative stress in *A. crenata,* represented by tissue catalase activity and lipid peroxidation, therefore, suggesting that chronic Cd exposure would have a deleterious effect on *A. crenata* health. Overall, the results of the present study demonstrate that *A. crenata* physiological and biochemical responses were tissue-specific, dose- and time-dependent, which may reflect its usefulness as a test organism for ecotoxicology studies.

Chapter 4

The intertidal mud snail, *Amphibola crenata* as a bioindicator of anthropogenic and natural stressors in the Avon-Heathcote Estuary/Ihutai

4.1 INTRODUCTION

Estuaries often receive anthropogenic metal inputs. Estuarine physicochemical processes can affect the bioavailability of those metals in both their dissolved and particulate form for organisms (Marsden et al., 2014). Sediment characteristics such as grain size, organic content and pH, chemical activities including dissolution and redox reactions, biological processes such as microbial activity along with currents and hydrology may all influence local bioavailability of trace metals to estuarine biota (De Gregori et al., 1996; Marsden et al., 2014; Zhang et al., 2014).

Trace metals can result in a number of impacts on organisms including acute or chronic toxicity, changes in population structure of benthic invertebrates, accumulate in biological tissues and biochemical/physiological alterations. Nutrient enrichment is also recognized as a serious threat to estuarine ecosystems worldwide (Wassmann 2005; Bricker et al. 2008). Nutrient inputs may result in increasing frequencies and magnitudes of hypoxic events, cause behavioural and physiological adaptations or avoidance and migratory responses of estuarine organisms (Marsden and Baharuddin, 2014). In addition, large scale catastrophic events like earthquakes can cause adverse impacts on estuarine organisms due to alterations of their habitat characteristics following such events. Some estuarine species, however, such as gastropods are able to adapt to those extreme environmental conditions and it might be expected that biological aspects of these species would reflect the environmental conditions (Cardoso et al., 2002; Marsden and Swinscoe, 2014), thus, usually can be used as good indicators of environmental health (Cardoso et al., 2013). Among aquatic organisms, intertidal communities are some of the organisms most affected by anthropogenic and natural disturbances and may exhibit cellular, individual, and population-level changes. Therefore, those intertidal organisms such as *A. crenata* could good indicator of the effect such disturbances have on aquatic biota.

4.1.1 Avon-Heathcote Estuary

The Avon-Heathcote Estuary/Ihutai is a shallow, predominantly intertidal estuary located in east to Christchurch city, New Zealand. The Estuary is the meeting point of two rivers, the Avon and the Heathcote, which drain a large part of the City of Christchurch. Significant amounts of contaminants, including trace metals and nutrients enter to the Estuary (Stevenson, 2010). Until

recently the Estuary received treated domestic and industrial effluents from a city storm water drain and a sewage treatment station (Marsden and Swinscoe, 2014); However, since the construction of an ocean outfall in 2010, nutrient supply to the Estuary has been reduced by approximately 90% (Skilton, 2013). In addition, the Estuary experienced a number of high magnitude earthquakes during 2010 – 2012, which had major impacts on estuarine characteristics including soft-sediment deformation and coseismic uplift in parts of the Estuary (Reid et al. 2012). All of these anthropogenic and natural disturbances may cause short or long-scale changes in estuarine biota, and thus may influence the overall health of the estuarine environment.

4.1.2 *Amphibola crenata* in the Avon-Heathcote Estuary

Amphibola crenata is a dominant macrofauna inhabited in intertidal areas of the Avon-Heathcote and widely distributed throughout the Estuary (Bennington, 1979; Marsden and Baharuddin, 2014). Populations of *A. crenata* have been reported in the Estuary for more than 30 years (Marsden and Knox, 2008). Previous studies on distribution of *A. crenata* in the Avon-Heathcote Estuary have shown that individual size increases towards the low-tide mark (Briggs, 1972; Bennington, 1979) However, recent studies have revealed that the distribution of *A. crenata* may varied within the estuary based on combination of factors including food availability and quality, salinity, water temperature, contaminant level and sediment conditions (Baharuddin, 2010; Marsden and Swinscoe, 2014).

4.1.2 Objectives

The aim of this study was to assess *A. crenata* as a potential bioindicator for both anthropogenic and natural stressors using physiological, biochemical and population level biomarker responses. The main objectives of this study were to 1) assess metal content in the soft body tissues of *A. crenata* as an indicator of sediment trace metal levels (e.g. As, Cd, Cu, Ni, Pb and Zn), 2) quantify biomarker responses including condition index (CI), glutathione-S-transferase (GST) activity, population structure and reproductive success of *A. crenata* in relation to the nutrient and metal content of the sediment, 3) evaluate the affect of sediment organic matter and grain size on trace metal availability, and 3) re-evaluate the post-earthquake population attributes of *A. crenata* (e.g.

density and length distribution), condition index (CI) and sediment conditions of the Avon-Heathcote Estuary compared with the 2008/2009 study by Nursalwa Baharuddin.

4.2 METHODS

4.2.1 Study area

The Avon-Heathcote Estuary is a shallow, micro-tidal estuary with a tidal range of 0-2 m (Deely and Fergusson, 1994). The Estuary is fed by the Avon and the Heathcote rivers at its northern and southwest margins. Nearly 80% of the Estuary is covered by mudflats, which are exposed during low tide (Marsden and Swinscoe, 2014). Sampling was undertaken in eight contrasting areas inhabited by *A. crenata*, with different levels of anthropogenic pressure (Table 4.1; Fig. 4.1). The Avon, Heathcote, Ferrymead, and Oxidation pond sites were historically contaminated. The Avon, Heathcote and Ferrymead sites were close to the rivers and the Oxidation pod site was close to the water treatment outfall (Bolton-Ritchie, 2015). The Tern site was located near the Estuary mouth, therefore is considered relatively uncontaminated (Marsden and Baharuddin, 2014) and used as a reference site for this study. All of the sites examined in the present study have been irregularly monitored for a long time, and sediment properties, trace metal characteristics of the sediment and macro- and micro-benthos community composition have been documented in various scientific reports and papers (Bennington, 1979; Deely and Fergusson, 1994; Marsden and Maclaren, 2010; Bolton-Ritchie, 2015).

Table 4.1 Habitat characteristics for locations used for the population structure survey of *A. crenata* and samples collected for the biomarker assays. Sites names are shown in Fig. 4.1.

Site	GPS Location	Sediment type	Salinity (‰)	Water temperature (° C)	Contaminant inputs and status
TS	S43° 33.136' E172° 44.709'	Fine sand, very fine sand, silt	28.4	23.5	Away from wastewater and river discharge. Clean site
GD	S43° 32.463' E172° 44.455'	Fine sand	28.7	28.5	Away from wastewater and river discharge. Post-earthquake damage
JT	S43° 32.007' E172° 43.857'	Fine sand	25.2	20.9	Influenced by oxidation ponds and river discharge
OP	S43° 32.542' E172° 43.234'	Fine sand, very fine sand, silt	20.9	14.2	Wastewater seepage from oxidation pons
FM	S43° 33.384' E172° 42.413'	Fine sand, very fine sand	23.4	13.5	River and industrial effluents
HT	S43° 33.657' E172° 42.478'	Fine sand, very fine sand	20.2	22.6	City and industrial effluents
MC	S43° 33.295' E172° 42.445'	Clay, silt	31.3	26.6	Domestic discharge and stormwater runoff from port Hills
AV	S43° 31.186' E172° 43.724'	Clay, silt	16.3	17.4	City discharge from Avon River

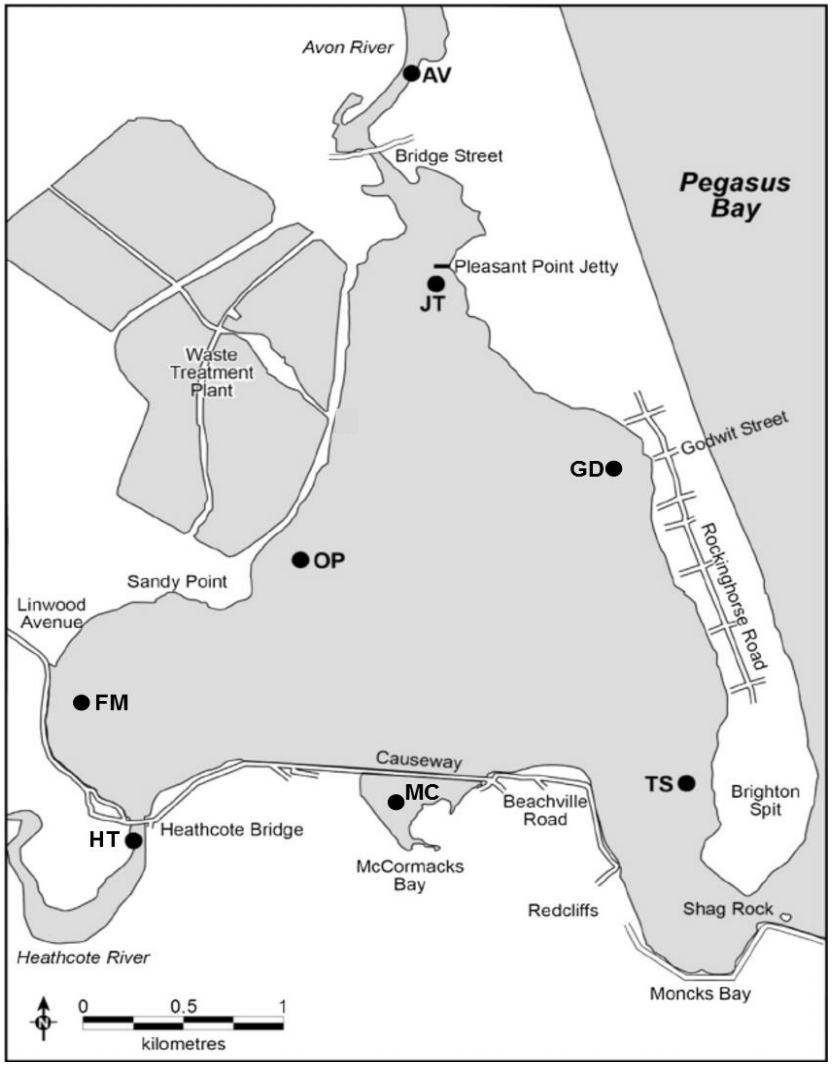


Figure 4.1 Map of the location of eight sampling sites in the Avon-Heathcote Estuary. TS, Tern; GD, Godwit; JT, Pleasant Jetty; OP, Oxidation ponds; FM, Ferrymead; HT, Heathcote; MC, McCormacks Bay; AV, Avon.

4.2.2 Sample collection and maintenance

Adult mud snails (n=20 per site; shell length > 17.9 mm) were randomly collected by hand at the mid tide from eight sampling sites in December 2016 (Fig. 4.1). They were transferred into labelled polyethylene containers with seawater from the collection site and transported alive to the laboratory at the University of Canterbury. In the laboratory mud snails were washed with sea water and maintained for 24 h in fresh seawater that had been collected from each study site to

void the sediment from the digestive tract. Subsequently the flesh was separated from the shell and stored at - 80 °C until further analysis. Sediment samples from surface layer (< 2 cm depth; n = 4) were collected from each site and placed in 70 ml polypropylene containers. On reaching the laboratory, samples were stored at -20 °C until processed.

4.2.3 Tissue and sediment trace metal analyses

After thawing, tissue samples (n = 8) from each study site were dried at 60 ° C for 72 h and dry weights were recorded. Weighed samples were then transferred into acid-washed tubes and acid digested overnight using 0.5 ml 70% ultrapure HNO_3. Digested samples were heated at 85°C for 1 hour. Once cool, each sample was diluted appropriately by adding milli-Q water. Trace metals of arsenic (As), cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb) and zinc (Zn) in the mud snail tissues were measured using inductively coupled plasma mass spectrophotometry (ICP-MS, Agilent-7500cx). Certified mussel tissue SRM 2976 (National Institute of Standards and Technology, US) was used to achieve the QA/QC standards. The mean ± SD recovery of the SRM for As, Cd, Cu, Ni, Pb and Zn were 109 ± 4%, 92 ± 9%, 100 ± 2%, 79 ± 11%, 81 ± 5% and 93 ± 4% respectively. The tissue trace metal was expressed as μg g dry wt.$^{-1}$

To determine trace metal content in the sediment, sediment samples were dried at 60 °C for 72 h and ground to obtain uniform grain sediment. Approximately 1g of each sample was weighed out into acid washed polycarbonate falcon tubes. 4 ml of HNO_3 (1:1) and 10 ml of HCl (1:4) acid was added and left overnight for facilitate acid digestion. Samples were then refluxed at 85°C in a heating blocks for 40 mins. Once cooled, final volume of the samples was made up to 20 ml with milli-Q water, left overnight to allow the particles to settle out. Samples were then diluted appropriately by adding 2% HNO_3 and analysed using ICP-MS (Chandurvelan et al., 2015). Certified marine sediment was used to achieve the QA/QC standards. The mean ± SD recovery of the SRM for As, Cd, Cu, Ni, Pb and Zn were 88 ± 9%, 94 ± 10%, 84 ± 11%, 65 ± 14%, 88 ± 7% and 90 ± 12% respectively. The sediment trace metal was expressed as mg kg^{-1}.

4.2.4 Metal Pollution Index (MPI)

At each site, metal pollution index was calculated for both the sediment and the snail whole body tissue as follows (Benson et al., 2016),

$$MPI = [C_1 \times C_2 \times C_3 \times \ldots\ldots \times C_n]^{1/n}$$

Where *n* is the number of metals and $C_n$is the concentration of metal *n* in sediment/*A. crenata* whole body tissue on dry weight basis.

4.2.5 Sediment total recoverable phosphorus (TRP), total nitrogen (TN), and organic matter (SOM)

A single sediment sample from the surface layer (to a ~ 2cm depth) was taken from each location. These samples were kept in a chilly-bin with ice and transported to the laboratory, kept at – 20 °C until further analysis at Hill Laboratories. Total recoverable phosphorus (TRP) was analysed using nitric/hydrochloric acid digestion, ICP-MS, screen level (US EPA 200.2). Total nitrogen (TN) was analysed using catalytic combustion, separation and thermal conductivity. Sediment organic matter (SOM) was estimated by combustion at 450 °C for 4 hrs.

4.2.6 Sediment grain size

At each site, surface sediments were collected with a hand held plastic scoop, and placed in plastic containers (n = 4). In the laboratory samples were dried at 60°C for 72 h and ground to obtain uniform grain sediment. Ground up samples were separated into particle size fractions, using a series of nylon and nytal mesh sieves. The nominal size range of sediments were: coarse sand > 500 μm; medium sand > 250 μm; fine sand > 125 μm; very fine sand > 63 μm and clay/silt < 63 μm.

4.2.7 Population structure

Population structure of *A. crenata* (density and length) was determined at mid-tide level for each site using the method described by Baharuddin (2010). At each of the sites, a 30 x 15 m plot was marked, and the GPS coordinates of each corner and the mid-point was recorded. Each plot was subdivided into four 15 x 7.5 m plots. Within each subplot, five random quadrats of 0.5 x 0.5 m were placed, and the length and density of all snails within the quadrat were recorded. To collect juvenile *A. crenata*, the quadrat area was dug to a depth of ~ 5 cm and sieved through a 1 mm mesh. Out of the eight sites sampled in the current study, five sites are identical to the sites (i.e. TS, JT, OP, FM and AV) used by Baharuddin (2010) which allows to compare the pre- and post-earthquake distribution of *A. crenata* population in the Avon-Heathcote Estuary.

4.2.8 Reproductive success

The numbers of reproducing snails and egg collars found within each plot (30 x 15 m; 450 m^2) were counted. This was done by dividing each plot into 10 columns (3 x 15 m) and slowly walking up and down along each column, egg collars and reproducing snails were noted while walking. When there were large numbers of egg collars and reproducing snails, random quadrat (0.5 x 0.5 m) sampling was used (20 quadrats per plot).

4.2.9 Condition Index (CI)

The dry weight condition index (CI) was measured for adult *A. crenata* collected from each study site (n=9). Each mud snail was wrapped in labeled tin foil and oven dried at 60 °C for 72 hours. After recording resulting dry weight (g), samples were transferred to an ashing oven and heated for 4 hours at 500 °C. Once cool, the dry tissue weight (g) of each individual was recorded and CI was calculated as follows (Wilson 1988; Marsden and Swinscoe, 2014),

$$Condition\ Index\ (CI) = \frac{Flesh\ weight\ (g)}{Shell\ weight\ (g)}$$

4.2.10 Glutathione-S-transferase (GST)

To determine GST activity, tissue samples (soft body tissue; n=6) were first homogenised in 800 µL of ice-cold homogenised buffer (100 mM Trizma base, 2 mM EDTA, 5 mM $MgCl_2.6H_2O$). The resulting homogenate was centrifuged at 30,000 ×g for 10 min at 4°C and 10 ml of supernatant was diluted by 30 × with the buffer. Tissue GST level was measured using glutathione-S-transferase assay kit (Sigma Aldrich, CS0410) following the method described by Keen et al., (1976). The method based on the conjugation of L-glutathione (GSH) to 1-Chloro-2,4-dinitrobenzene (CDNB) through the thiol group of the glutathione with the presence of GST. The absorbance of GS-DNB conjugate was measured at 340 nm using 96 well UV plate reader, which is directly proportional to the GST activity of the sample. The tissue GST activity was expressed as µmol/g wet wt./min.

4.2.11 Statistical analyses

All data were processed using R statistical software (R version 3.0.2). Trace metal concentrations in the sediment and tissues and the different biomarker responses were compared between sites using a one-way ANOVA followed by a post-hoc test (TukeyHSD). The Pearson correlation (R) was conducted to determine the relationship between environment variables and environment variables to tissue trace metals and different biomarker responses. Principal component analysis (PCA) was used to understand the relationships between the mud snail population distribution and environment parameters. A value of $p < 0.05$ was considered statistically significant.

4.3 RESULTS

4.3.1 Sediment trace metals, nutrients, and organic matter

Surface sediments contained low levels of trace metals (Table 4.2), arsenic (1.8 – 6.5 mg/kg), cadmium (0.02 – 0.1 mg/kg), copper (1.8 – 13.3 mg/kg), nickel (6.4 – 13.1 mg/kg), lead (5.5 – 27.6 mg/kg) and zinc (29.5 – 96. 7 mg/kg). The trace metals, however, showed a clear spatial gradient. For most of the metals, values were highest for AV > MC > HT > FM > OP, and values were relatively low and comparable at TS, GD and JT. The AV site showed significantly higher

levels for all trace metals ($p < 0.05$) tested, while MC site exhibited significantly higher concentrations of Cu, Zn, Cd and Pb ($p < 0.05$) compared to the reference site of TS. The levels of sediment trace metals were all below than Interim Sediment Quality Guidelines-Low (ISQG-Low) as determined by the Conservation Council and the Agriculture and Resource Management Council of Australia and New Zealand (ANZECC, 2000) for all sampling sites.

Site AV had the highest concentrations of total recoverable phosphorous (TRP) and total nitrogen (TN), followed by MC. All other sites had relatively low levels of TRP ranging from 300 – 420 mg/kg and TN levels below 0.05 g/100g. Considering the sediment nutrient values, AV and MC sites had an 'enriched' condition rating for TRP and a 'low-moderate' condition rating for TN, while all other sites had a 'low-moderate' condition rating for TRP and a 'very good' condition rating for TN (Williamson et al., 2017). Sediment TRP increased with As ($R = 0.91$; $p < 0.05$), Cd ($R = 0.81$; $p < 0.05$), Cu ($R = 0.99$; $p < 0.01$), Ni ($R = 0.96$; $p < 0.05$), Pb ($R = 0.98$; $p < 0.01$) and Zn ($R = 0.96$; $p < 0.05$; Table 4.5). Sediment organic matter (SOM) was variable among sites with highest value recorded at AV (19.2 g/kg) and lowest at GD (6.3g/kg; Table 2), in order AV > MC > FM > TS > OP > HT > JT > GD. The SOM significantly increased with sediment trace metals and TRP (Table 4.5).

4.3.2 Metal Pollution index (MPI)

MPI values estimated based on both the sediment trace metals and snail tissue trace metals are presented in Table 4.3. Values are ranged between 2.3 to 8.5 in terms of the sediment trace metals, whereas values are ranged between 5.8 to 8.3 in terms of the tissue trace metals. Both methods estimated highest MPI values for the AV and MC sites. In addition, MPI values between sediment and the *A. crenata* tissue showed a significant positive correlation ($R^2 = 0.727$; $p < 0.05$; Fig. 4.2).

Table 4.2 Trace metals (expressed as mg/kg; n = 4), total recoverable phosphorus (TRP; n = 1), total nitrogen (TN; n = 1) and sediment organic matter (SOM; n= 4) concentrations of sediment samples collected from eight different sites in the Avon-Heathcote Estuary. For trace metals, data are presented as mean ± SEM. Values with different letters (superscript) indicate significant differences between sampling sites ($p < 0.05$). Site names are shown in Fig. 4.1.

Site	Cu	Zn	Ni	As	Cd	Pb	TRP (mg/kg)	TN (g/100g)	SOM g/kg
TS	2.69 ± 0.07^{a}	29.68 ± 0.50^{a}	7.79 ± 0.14^{a}	2.36 ± 0.10^{acb}	0.02 ± 0.001^{a}	6.00 ± 0.10^{a}	350	< 0.05	9.1^{a}
GD	1.8 ± 0.06^{a}	30.06 ± 0.58^{a}	6.49 ± 0.13^{a}	1.87 ± 0.08^{ac}	0.04 ± 0.001^{a}	5.53 ± 0.16^{a}	300	< 0.05	6.3^{a}
JT	1.96 ± 0.01^{a}	29.53 ± 0.40^{a}	6.43 ± 0.10^{a}	2.71 ± 0.05^{acb}	0.02 ± 0.001^{a}	6.53 ± 0.10^{a}	330	< 0.05	7.9^{a}
OP	2.58 ± 0.06^{a}	35.67 ± 0.67^{a}	6.87 ± 0.11^{a}	2.06 ± 0.03^{c}	0.06 ± 0.002^{ca}	7.03 ± 0.09^{a}	420	< 0.05	9.0^{a}
FM	3.55 ± 0.28^{a}	42.11 ± 2.76^{a}	7.47 ± 0.43^{a}	2.97 ± 0.18^{b}	0.05 ± 0.007^{ca}	8.22 ± 0.49^{a}	370	< 0.05	9.4^{a}
HT	3.7 ± 0.03^{a}	48.56 ± 2.70^{ab}	7.2 ± 0.16^{a}	3.33 ± 0.08^{db}	0.09 ± 0.009^{cd}	10.38 ± 0.20^{a}	380	< 0.05	8.7^{a}
MC	7.83 ± 1.52^{a}	73.12 ± 11.19^{b}	8.43 ± 0.84^{a}	3.16 ± 0.37^{db}	0.11 ± 0.02^{db}	17.86 ± 3.39^{b}	640	0.13	18.0^{b}
AV	13.31 ± 0.29^{c}	96.74 ± 2.61^{b}	13.13 ± 0.33^{b}	6.47 ± 0.16^{e}	0.12 ± 0.008^{b}	27.59 ± 0.57^{d}	990	0.14	19.2^{b}

Table 4.3 MPI values for the sediment and *A. crenata* collected from eight sites in the Avon-Heathcote Estuary.

Site	MPI	
	Sediment	Tissue
TS	2.4	6.8
GD	2.3	6.0
JT	2.3	5.8
OP	2.8	5.8
FM	3.3	6.1
HT	3.3	6.5
MC	5.5	8.3
AV	8.5	8.2

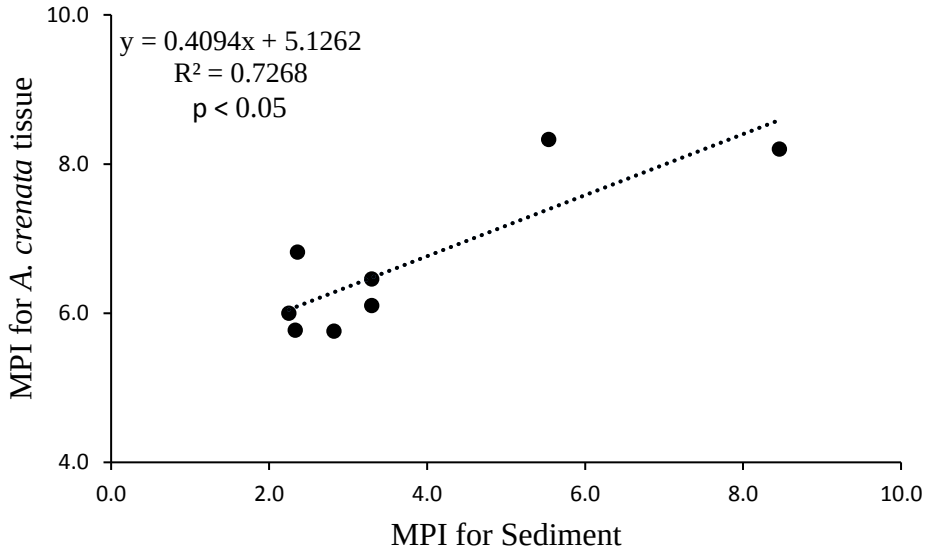


Figure 4.2 Relationship between sediment and *A. crenata* tissue MPI values.

4.3.3 Sediment grain size

The grain size distribution of sediments in the sampling sites is presented in Fig 4.3. The < 63 μm (clay/silt) size was the dominant fraction in AV and MC sites, accounting for 61 – 66% of the sediments, while 125 – 250 μm (fine sand) size was the dominant fraction in all other sites,

ranging from 42 – 77% of the sediments. The amount of the > 500 μm (coarse sand) was the smallest fraction with values ranging from 0.4 – 7%.

The sediment trace metals (As, Cd, Cu, Ni, Pb, Zn and total metal in sediment), TRP and SOM were significantly correlated with the clay/silt (< 63 μm) fraction of the sediment and was inversely correlated with the fine sand (125 – 250 μm) fraction (Table 4.6).

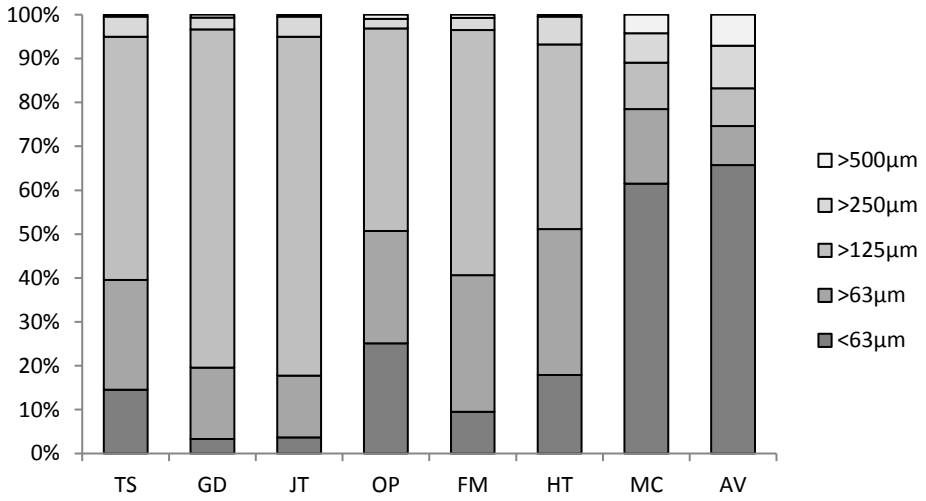


Figure 4.3 Grain size distribution of sediments in sampling sites (by %). The nominal size range of sediments were: coarse sand > 500 μm; medium sand > 250 μm; fine sand > 125 μm; very fine sand > 63 μm and clay/silt < 63 μm.

4.3.4 Trace metals in mud snail tissues

Trace metal concentrations in mud snail tissues had a wide distribution (Table 4.4), As (9.7-24.3 mg/kg), Cd (0.11-.39 mg/kg), Cu (58-102 mg/kg), Ni (2.8-3.8 mg/kg), Pb (2.5-4.7 mg/kg) and Zn (35-48 mg/kg). Concentrations of Cu in the tissues exceeded levels of Zn and As, while low levels of Ni, Pb, and Cd were measured. The total metal concentration in the tissues followed the expected pollution gradient ($R = 0.83$; $p < 0.05$) with the highest values recorded at the AV site. For most of the trace metals except Ni and As, the tissue concentration had a significant positive correlation ($p < 0.05$) with sediment trace metals and SOM (Table 4.6).

Table 4.4 Trace metals (expressed as µg/g dry wt.; n = 8) concentration of mud snail tissue samples collected from eight sites in the Avon-Heathcote Estuary. Data are presented as mean ± SEM. Values with different letters (superscript) indicate significant differences ($p < 0.05$).

Site	Metals (µg/g dry wt.)					
	Cu	Zn	Ni	As	Cd	Pb
TS	62.35 ± 3.5^{a}	39.05 ± 2.0^{a}	2.92 ± 0.4	22.05 ± 1.6^{a}	0.19 ± 0.02^{ab}	3.39 ± 0.2^{a}
GD	72.39 ± 5.8^{a}	39.68 ± 3.3^{ab}	2.77 ± 0.1	21.47 ± 0.7^{a}	0.11 ± 0.01^{a}	2.52 ± 0.1^{a}
JT	62.28 ± 2.6^{a}	35.07 ± 0.5^{a}	3.12 ± 0.1	11.73 ± 0.4^{b}	0.15 ± 0.04^{ab}	3.19 ± 0.1^{a}
OP	62.70 ± 4.1^{a}	41.93 ± 1.3^{ab}	3.25 ± 0.1	12.51 ± 0.6^{b}	0.13 ± 0.01^{a}	2.54 ± 0.1^{a}
FM	63.19 ± 1.8^{a}	46.13 ± 1.1^{b}	3.36 ± 0.3	9.71 ± 0.5^{b}	0.16 ± 0.01^{ab}	3.43 ± 0.2^{a}
HT	58.40 ± 4.0^{a}	42.05 ± 1.9^{ab}	3.57 ± 0.5	10.95 ± 0.2^{b}	0.25 ± 0.02^{bd}	3.03 ± 0.2^{a}
MC	74.15 ± 6.0^{a}	47.06 ± 4.3^{b}	3.02 ± 0.2	24.34 ± 1.7^{a}	0.39 ± 0.02^{c}	3.35 ± 0.3^{a}
AV	101.58 ± 11.8^{c}	48.63 ± 1.9^{b}	3.77 ± 0.3	12.81 ± 1.3^{b}	0.27 ± 0.03^{d}	4.67 ± 0.6^{b}

4.3.5 Population structure

Marked differences in the population structure were observed at the eight sampling sites with both unimodal (TS, GD, OP, HT, MC and AV) and bimodal (JT and FM) distributions (Fig. 4.4). Considering the density distribution of *A. crenata* (Fig. 4.5), the AV site had the highest value (86 ind/m^2) followed by MC (60 ind/m^2), while GD had the lowest value (20 ind/m^2). The TS, GD, OP, HT and MC sites were dominated by large individuals (> 17.9 mm), while JT and FM sites were dominated by both small (< 7.9 mm) and medium (7.9 – 17.9 mm) individuals. The AV site was dominated by medium individuals. The density of medium individuals was positively correlated ($p < 0.05$) with sediment trace metals (As, Cu, Ni, Pb, Zn and total metal in sediment), TRP and SOM (Table 4.6).

4.3.6 CI and GST

For most of the contaminated areas, the CI of adult *A. crenata* exhibited low values (AV < OP < FM < HT < MC), ranging from 0.17 – 0.21 and showed significant decline ($p < 0.05$) compared to the reference site of TS, while high CI values were observed for areas with relatively low contamination (JT < GD < TS), ranging from 0.32 – 0.33 (Fig. 4.6A).

The GST levels in *A. crenata* were higher in the contaminated areas (FM < HT < AV < OP < MC) and ranging from 7.2 – 15.8 µmol/g wet wt./min with significantly high values ($p < 0.05$) observed for AV, OP and MC sites compared to the reference site of TS. Reduced GST activity was observed in relatively clean areas (GD < TS < JT), ranging from 6.6 – 6.8 µmol/g wet wt./min (Fig. 4.6B).

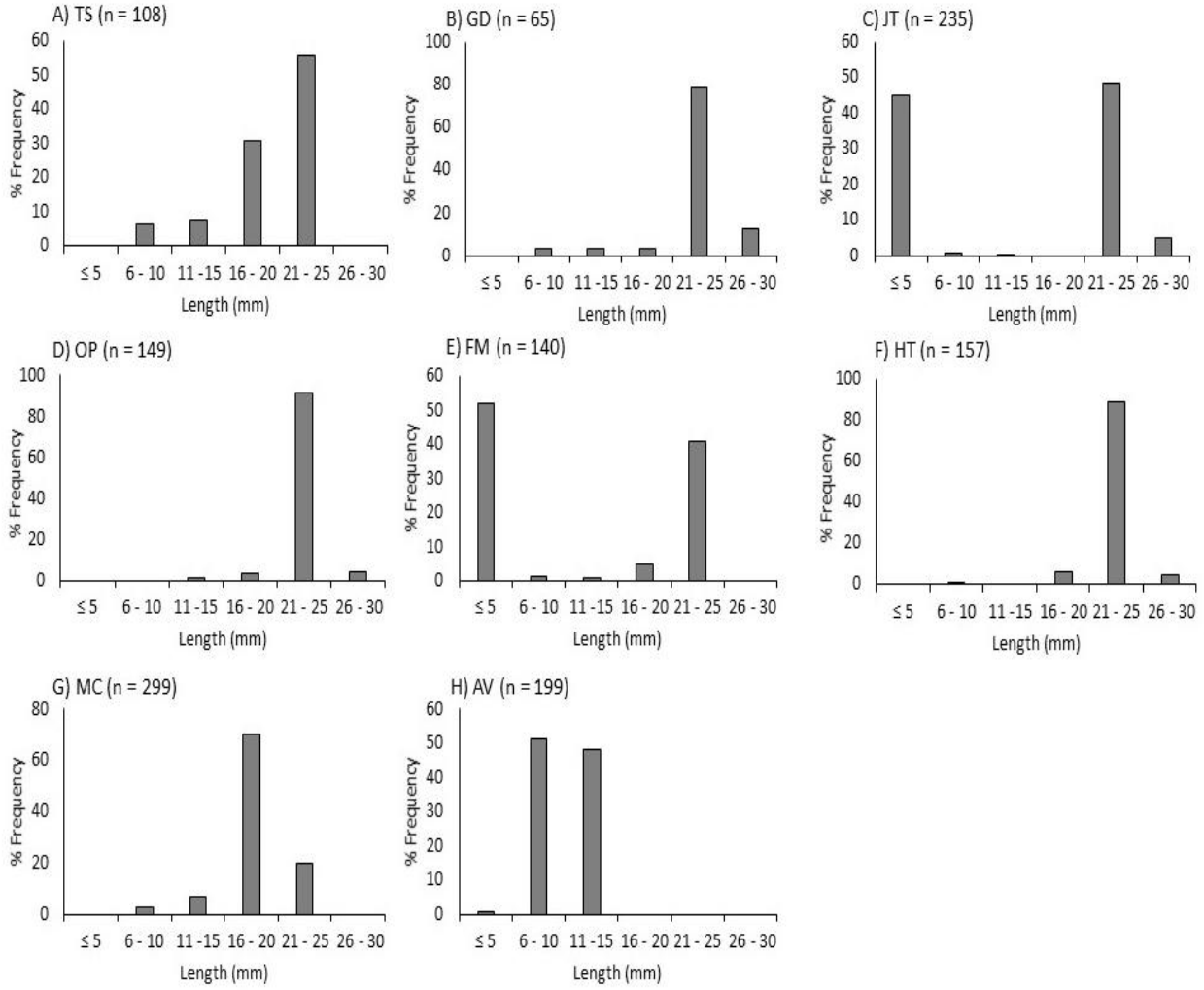


Figure 4.4 Length distribution (% frequency) of *A. crenata* in eight sites (mid-tide) in Avon-Heathcote Estuary.

4.3.7 Relationship of sediment grain size to metal bioaccumulation and biomarker responses

The concentration of Cu, Zn, Cd and total metal in tissue (TMT) increased with the increasing clay/silt fraction of sediment and was negatively correlated with the fine sand. The CI had a significant positive correlation ($R = 0.72$; $p < 0.05$) with the sand fraction of the sediment, while the GST had a significant positive correlation ($R = 0.84$; $p < 0.05$) with clay/silt fraction of the sediment.

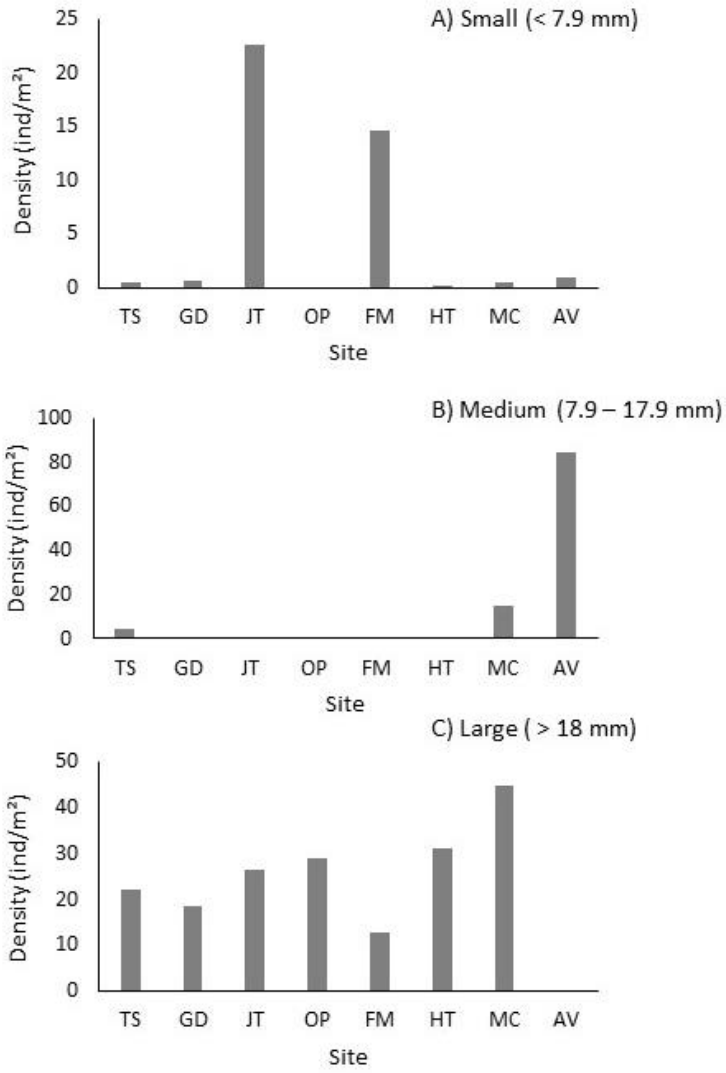


Figure 4.5 Density of small (< 7.9 mm) (A); medium (7.9 – 17.9 mm) (B); and large (> 17.9 mm) (C) A. crenata from eight sites in the Avon-Heathcote Estuary.

4.3.8 Reproductive success

The numbers of reproducing snails and egg collars were variable between sites (Table 4.5). The highest value of reproducing snails was recorded from OP and lowest at GD, in order OP > FM > JT > HT > GD, while the highest number of egg collars were recorded from GD followed by JT and HT. Noticeably, no reproducing snails or egg collars were found in TS, MC and AV sites.

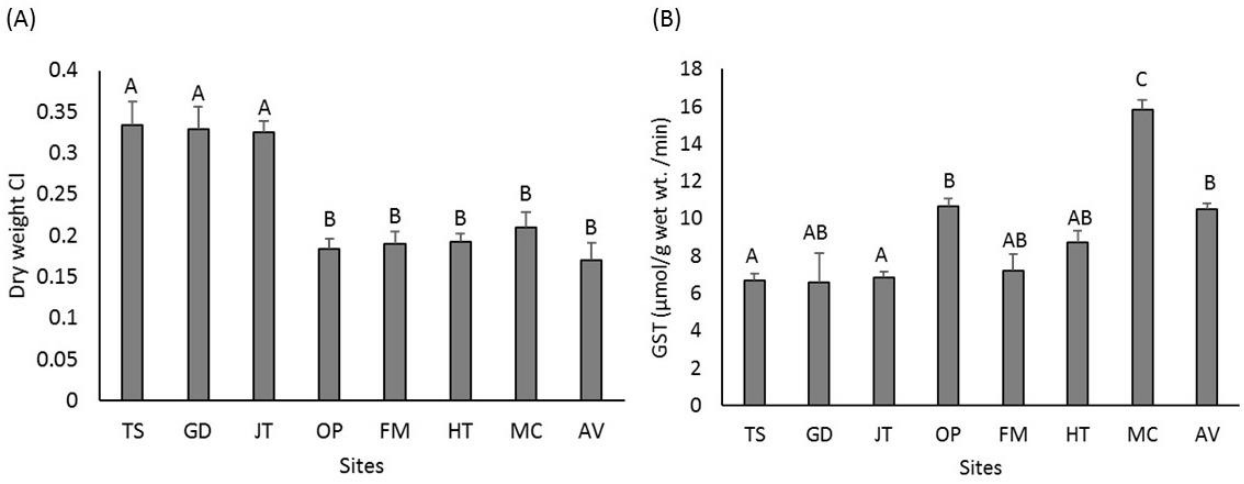


Figure 4.6 Biomarker measurements of CI (A); and GST (B) of *A. crenata* collected from eight sites in Avon-Heathcote Estuary. Data are presented as mean ± SEM (n = 9 for CI; 6 for GST). Means with different letters significantly differ at $p < 0.05$.

Table 4.5 Number of reproducing snails and egg collars in the sampling sites of the Avon-Heathcote Estuary. The area represented by each sample site was 450 m^2.

Site	Number of reproducing snail	Number of egg collar
TS	0	0
GD	20	160
JT	29	91
OP	53	0
FM	47	0
HT	24	53
MC	0	0
AV	0	0

Table 4.6 Correlation (R) analysis of *A. crenata* biomarker responses with environmental parameters. Values in bold indicate significant correlation ($p < 0.05$). TMS, total metal in sediment; TRP, total recoverable phosphorous; SOM, sediment organic matter; C/S, clay/silt fraction; Sand/f, fine sand fraction TMT, total metal in tissue; CI, dry weight condition index; GST, glutathione-S-transferase; S, M and L, density of small, medium and large A. crenata; RPS, reproducing snail; EC, egg collar.

	Sediment											*A. crenata*													
	Cu	Zn	Ni	As	Cd	Pb	TMS	TRP	SOM	C/S	Sand/f	Cu	Zn	Ni	As	Cd	Pb	TMT	CI	GST	S	M	L	RPS	EC
Cu	1.00																								
Zn	**0.98**	1.00																							
Ni	**0.96**	**0.90**	1.00																						
As	**0.93**	**0.89**	**0.95**	1.00																					
Cd	**0.87**	**0.94**	**0.73**	**0.76**	1.00																				
Pb	**0.99**	**0.99**	**0.93**	**0.92**	**0.90**	1.00																			
TMS	**0.99**	**1.00**	**0.93**	**0.92**	**0.91**	**1.00**	1.00																		
TRP	**0.99**	**0.96**	**0.96**	**0.91**	**0.84**	**0.98**	**0.98**	1.00																	
SOM	**0.95**	**0.95**	**0.85**	**0.76**	**0.85**	**0.94**	**0.94**	**0.93**	1.00																
C/S	**0.91**	**0.93**	**0.81**	**0.72**	**0.89**	**0.92**	**0.92**	**0.92**	**0.97**	1.00															
Sand	**-0.87**	**-0.91**	**-0.77**	**-0.72**	**-0.91**	**-0.88**	**-0.89**	**-0.86**	**-0.90**	**-0.96**	1.00														
Cu	**0.90**	**0.84**	**0.91**	**0.83**	0.66	**0.88**	**0.86**	**0.91**	**0.83**	**0.75**	**-0.72**	1.00													
Zn	**0.78**	**0.83**	0.70	0.64	**0.82**	**0.77**	**0.81**	**0.75**	**0.78**	**0.78**	**-0.84**	0.61	1.00												
Ni	0.61	0.63	0.63	**0.79**	0.66	0.63	0.64	0.61	0.38	0.44	-0.55	0.40	0.55	1.00											
As	0.01	0.00	-0.05	-0.28	-0.05	-0.01	-0.01	0.00	0.26	0.21	-0.11	0.10	-0.03	**-0.73**	1.00										
Cd	0.69	**0.77**	0.52	0.51	**0.79**	**0.72**	**0.73**	0.64	**0.80**	**0.83**	**-0.86**	0.39	0.62	0.29	0.33	1.00									
Pb	**0.86**	**0.79**	**0.91**	**0.92**	0.56	**0.83**	**0.82**	**0.82**	**0.73**	0.65	-0.63	**0.75**	0.57	0.62	-0.15	0.50	1.00								
TMT	**0.89**	**0.85**	**0.86**	**0.72**	0.70	**0.87**	**0.86**	**0.88**	**0.91**	**0.83**	**-0.72**	**0.94**	**0.71**	0.24	0.36	0.56	0.69	1.00							
CI	-0.57	-0.65	-0.47	-0.53	-0.70	-0.58	-0.62	-0.56	-0.49	-0.59	**0.72**	-0.30	**-0.81**	**-0.79**	0.47	-0.45	-0.35	-0.30	1.00						
GST	0.57	0.66	0.36	0.29	**0.75**	0.61	0.62	0.59	**0.75**	**0.84**	**-0.82**	0.35	0.63	0.16	0.32	**0.82**	0.20	0.51	-0.57	1.00					
S	-0.29	-0.32	-0.29	-0.10	-0.44	-0.28	-0.30	-0.30	-0.34	-0.45	0.50	-0.27	-0.39	-0.01	-0.48	-0.35	0.04	-0.44	0.25	-0.41	1.00				
M	**0.94**	**0.87**	**0.98**	**0.94**	0.70	**0.92**	**0.90**	**0.95**	**0.81**	**0.76**	**-0.68**	**0.95**	0.61	0.61	-0.08	0.42	**0.87**	**0.86**	-0.41	0.31	-0.24	1.00			
L	-0.36	-0.24	-0.56	-0.55	-0.02	-0.30	-0.30	-0.36	-0.12	0.00	-0.03	-0.56	-0.23	-0.43	0.39	0.36	-0.56	-0.37	0.08	0.49	-0.11	-0.60	1.00		
RPS	-0.26	-0.25	-0.25	-0.32	-0.11	-0.26	-0.26	-0.14	-0.25	-0.06	0.05	-0.25	-0.07	0.04	-0.28	-0.36	-0.46	-0.30	-0.32	0.15	-0.17	-0.22	0.17	1.00	
EC	-0.46	-0.45	-0.46	-0.37	-0.39	-0.42	-0.44	-0.46	-0.45	-0.56	0.68	-0.14	-0.59	-0.46	0.13	-0.48	-0.49	-0.23	0.62	-0.48	0.18	-0.31	0.00	-0.23	1.00

4.4 DISCUSSION

In the past, sediment trace metal levels in the Avon-Heathcote Estuary have been quite high due to inputs from treated sewage, but these have declined recently due to the diversion of the outfall directly to the ocean (Bolton-Ritchie, 2011), together with exchange of old sediment by new sediment due to liquefaction following the 2010 – 2012 earthquake disturbances (Cochran al., 2014). The sediment trace metals in the Avon-Heathcote Estuary, including all sampling sites of the present study, are below than ISQG-Low levels that the limits expected to show a biological effect (ANZECC, 2000). Sediment nutrients and SOM showed site-specific variation with greater values for the AV and MC sites. Therefore, it is likely that the biological effects observed in the present study for *Amphibola crenata* may result from the cumulative effects of multiple stressors, including trace metals, nutrients, organic matter, and the presence of other organic and inorganic contaminants (not measured), either in the sediment or in the surrounding water.

4.4.1 Sediment trace metals, nutrients, and organic matter

Considering the site-specific variation in sediment trace metals, nutrients and SOM, the highest concentrations of all elements were recorded at the AV site. These high concentrations may be attributed to waste discharge from the City of Christchurch. For most of the sites, the spatial distribution of sediment trace metals found in this study were generally similar to those found in previous studies (Marsden and Baharuddin, 2014). Overall, trace metal concentrations in the Avon-Heathcote Estuary sediment were found to be in the following descending order: Zn > Pb > Ni > Cu > As > Cd. The results indicate a significant positive interaction between sediment trace metals, TRP and SOM. Delaney (1998) reported that a large fraction of sediment TRP could be derived as organic phosphorus, which may explain the observed positive relationship between sediment TRP and SOM in the present study. Charriau et al. (2011) reported a strong positive association between sediment trace metals and SOM. Zhang et al. (2014) found that SOM plays a vital role for metal-binding in sediment. SOM can provide dissolved ligand for metals to form soluble complexes and SOM is believed to serve as a principle geo-sorbent for sediment trace metals, and could potentially trap large amounts of trace metals by sediment enriched with organic matter. This may explain the observed positive correlation between sediment trace metals and

SOM in the present study. The observed positive relationship of sediment trace metals, total recoverable phosphorous and organic matter to the finer fraction (< 63 µm) of the sediment may be attributed to the increase in the specific surface properties of this fraction which is consistent with the findings of De Gregori et al., 1996 and Martinez-Garcia et al., 2015.

4.4.2 Metal bioaccumulation in *Amphibola crenata*

The results indicated that *Amphibola crenata* readily accumulates trace metals from their surroundings and the concentration of trace metals in the tissues reflect environmental exposure, suggesting that metal accumulation in *A. crenata* can be used as an indicator of environmental contamination. Therefore, the results of the present study disagree with the findings of Bennington (1979), who suggested that *A. crenata* soft tissues may not represent the environmental metal concentration. Bennington (1979) suggests that large mud snails may able to regulate ion up take and excretion to maintain a low concentration of metals, the results based on limited number of surveys (4 study sites). In contrast, the present study demonstrates that trace metal accumulation in large-sized groups inhabited in different areas of the Estuary were comparable to sediment concentration. The general pattern of metal accumulation (Cu > Zn > As > Pd > Ni > Cd) in the present study was similar to those reported for other gastropods (Kupekar and Kulkarni, 2014).

Cu is an essential micronutrient for the growth and metabolism of living organisms (Aaseth and Norseth, 1986). Kupekar and Kulkarni (2014) have suggested that marine gastropods can accumulate and store Cu and utilise it in the synthesis of haemocyanin a haemolymph pigment. Previous study on the marine snail, *Murex brandaris* by Bouquegneau et al., (1984) found that the gastropod species have the ability to store Cu from the metabolism of haemocyanin as CuS in pore cells located in connective tissue of the digestive gland. Conti and Cecchetti (2003) also found high levels of Cu in the tissues of two marine gastropods, *Monodonta turbinate* and *Patella cerulea* in Tyrrhenian coastal areas and those authors concluded that high levels of Cu in gastropods tissues could be associated with high concentrations of water-soluble fraction of metals rather than metals incorporated with the sediment. Studies on the freshwater snail, *Pomacea paludosa* by Hoang and Rand (2009) suggests that gastropod species may require considerable quantities of carbonate for their shell development. Therefore, they may uptake carbonate as Cu carbonate from their surroundings and Cu carbonate may be isolated within the tissues through chemical and biological

reactions and carbonate would be used for shell development, meanwhile Cu would be accumulated in soft tissues. Therefore, all of these factors may be of some utility with respect to the observed high tissue Cu in *A. crenata* in the current study.

The lower accumulated levels of the most toxic elements (Pb, Ni and Cd; with the exception of As) suggests the presence of mechanisms that limit their accumulation such as excretory pathways and uptake limitation (Rainbow, 2002). High concentrations of arsenic appear to be present in tissues of certain marine species such as bivalve molluscs and herbivorous snails that feed primarily on microalgae and bacteria. Marine algae and bacteria often contain high levels of arsenic as they are able to accumulate dissolved arsenate from seawater (Neff, 1997), which may explain the observed relatively high concentration of As measured in deposit feeding *A. crenata* in the present study.

4.4.3 Metal Pollution index (MPI)

The MPI is used to simplify the data and to provide one value instead of many values when the measured metals are beyond five in number (Javed and Usmani, 2013). The MPI clearly indicates that the sites in the present study were distinguishable based on both the sediment and tissue MPI values. The positive relationship between the sediment and tissue MPI values highlighted that in *A. crenata* tissue trace metal is a valid measure of environmental metal concentrations. However, the observed differences between MPI values for the sediment and tissue in *A. crenata* can be explained by the other potential pathways (e.g. via food and/or water column) (Stankovic et al., 2015).

4.4.4 Relationship of *Amphibola crenata* population structure to environmental conditions

Previous studies on the population distribution of *A. crenata* have shown that they are abundant in the more contaminated parts of the estuary (Marsden and Swinscoe, 2014), which is in agreement with the findings of the present study; However, the results of the present study indicated that the medium-sized individuals (7.9 – 17.9 mm) were present in areas where trace metals, nutrients and SOM levels are high (e.g. AV and MC), whereas large individuals (> 17.9 mm) were relatively

evenly distributed among sites except for MC, suggesting that medium-sized *A. crenata* can opportunistically colonise in contaminated areas (Fig. 4.7). It is suggested that such responses may be associated with increased food availability and less competition from small/large individuals of their own species or other species in contaminated areas (Norkko et al., 2010; Marsden and Swinscoe, 2014). These results also corroborate the findings of Marsden and Baharuddin (2014), who noted that the highest survival rate of medium-sized *A. crenata* was where contaminant levels were relatively high. Bennington (1979) also noticed site-specific size frequency differences in *A. crenata* population in the Avon-Heathcote Estuary and concluded that these species may have evolved a strategy for partitioning the estuarine environment amongst different size classes to reduce intraspecific competition, increase sexual contact between breeding adults and maximize exploitation of the available resources

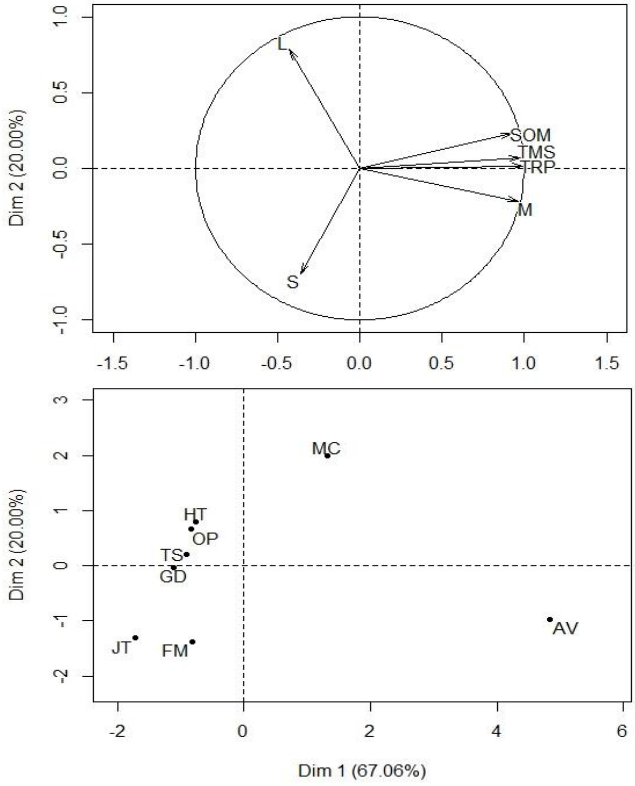


Figure 4.7 Principal component analysis (PCA) showing the relationship between *A. crenata* population structure and site-specific environmental variables. S, M and L, density of small, medium and large *A. crenata*; SOM, sediment organic matter; TMS, total metal in sediment; TRP, total recoverable phosphorous. PCA results suggested that the medium individuals are closely associate with the SOM, TMS and TRP.

The population structure of *A. crenata* could therefore be a useful bioindicator for estuarine contamination. Similarly, Cardoso et al. (2002) found increased abundance of juveniles of the mud snail, *Peringia ulvae*, in highly eutrophicated areas of the Mondego Estuary in Portugal. Another study by Johnson and Short (2013) has found higher densities of juvenile *Nassarius obsoletus* in nutrient enriched creeks in Massachusetts. Cardoso et al. (2013) noted high number of juveniles and young individuals of *Peringia ulvae* in the areas contaminated with mercury in the Ria de Aveiro Lagoon, Portugal.

4.4.5 Biomarker responses of *Amphibola crenata*

Variations in the physiological state of mud snails as shown by CI results (Fig. 4.6A) were in accordance with the pollution levels of the sites with low values found in areas where trace metals and nutrient levels were high. Relatively high CI values were recorded in areas with relatively low contaminants. In addition, CI of *A. crenata* appears to be negatively correlated with both sediment trace metals and TRP. This is in accordance with the findings of Marsden and Baharuddin (2014) in which the CI of *A. crenata* was negatively correlated with sediment trace metals, nitrogen and phosphorous levels. Similarly, the negative correlation between environmental trace metals and the dry weight condition index was previously found for other molluscs species such as mussels, *Mytilus galloprovincialis* (Rouane-Hacene et al., 2015) and *Perna viridis* (Yap and Al-Barwani, 2012). For Zn and Ni, there was a significant negative correlation between metal concentration in the tissue and the dry weight condition index of *A. crenata*. A similar negative relationship between CI and metal content of the tissues was previously found for the trace metals; Cu, Zn, Cd and Pb in other molluscs including bivalves and cockles (Reidel et al., 1998; Marsden et al., 2014). In contrast, some studies, however, demonstrated that CI of aquatic organisms may not always reflect the environmental contamination, but could be linked to species specific physiological functions such as spawning or sexual ripening (Lucas and Beninger, 1985; Rebelo et al., 2005). For example, Amiard et al., (2004) reported that the CI variations in mussel *Mytilus edulis* were linked to sexual ripening in mussel rather than trace metal contamination of the study area. Peake et al. (2006) found that tissue trace metal concentration was independent of CI in the cockle *Austrovenus stutchbury*, found in the southern New Zealand. Several studies have shown that many other biotic and abiotic factors

including age, size, and seasons also affect the CI of marine gastropods (Leung and Furness, 2001; Marsden and Swinscoe, 2014; Marsden and Baharuddin, 2014).

The GST are a group of antioxidant defense enzymes, which play a critical role in protecting the cells against damages caused by oxidative stress and peroxidative products (Van der Oost et al., 2003). A number of studies have shown that the presence of xenobiotic contaminants such as trace metals, pesticides, and insecticides in the environment induced GST activity in gastropod species (El-Shenawy et al., 2012; Bhagat et al., 2017). This is in accordance with the findings of the present study for *A. crenata*, where the elevated activity of GST was found in mud snails collected from contaminated areas. In the present study *A. crenata* tissue GST activity did not show a significant correlation with the trace metal levels in either sediment or tissues. Therefore, it is suggested that the relatively high levels of GST activity observed in *A. crenata* collected from the contaminated areas may be related to the presence of other xenobiotic substances such as organic contaminants in the area. For example, increased concentration of GST was found in the mussel, *Mytilus edulis* exposed to anthracene pollution (Yuan et al., 2017). Elevated levels of GST activity were found in the freshwater snail, *Brachmia purificata* collected from the areas contaminated with landfill leachate effluent and bisphenol A (Li et al., 2008). In contrast, GST activity was reduced in the marine snail, *Hexaplex trunculus*, when exposed to trace metal contaminants (Romeo et al., 2006) and no effects were found in the freshwater snail, *Planorbarius corneus* exposed to organophosphate insecticide chlorpyrifos (Rivadeneira et al., 2013), and *Biomphalaria glabrate* exposed to azinphos-methyl (Kristoff et al., 2006). Although the effect of anthropogenic stressors on tissue GST activity may vary greatly depending on the species and type of xenobiotic substance, a large number of literatures supports the positive relationship between organisms tissue GST activity and environmental contamination (Li et al., 2008; El-Shenawy et al., 2012; Cabecinhas et al., 2014; Leomanni et al., 2015; Bhagat et al., 2017)

Reproductive success determines fitness of an organism and reproductive impairment can cause long-term population level changes in organisms. *A. crenata* is a hermaphrodite snail, which lay eggs in a protected sand collar (nidus) with an estimate of 18,000 eggs per nidus. Adult snails can produce 12 to 15 egg collars per season (Pechenik et al., 2003). In the current study, in situ measurements of the number of reproducing snails and egg collars showed no clear trends in

relation to the levels of sediment trace metals, nutrients, organic matter and grain size. The reproductive behavior of this species may depend on a number of site-specific abiotic and biotic factors such as the strength of tidal current, height of the water column during high tide, salinity, pore water content of sediment, predatory pressure, inter and intra-specific competition and food availability. For example, the lack of egg collar at the TS site could be attributed to high tidal current, salinity and predatory pressure due to proximity to the estuarine mouth, while relatively high number of egg collars at the GD site may be attributed to low levels of contaminants, low strength of tidal current and optimum nutrient conditions in the area. Overall, the results suggest that governing factors of *A. crenata* reproductive performance are not only confined to the contaminants present in the environment but may also associate with many other biotic and abiotic factors, complicating the task of assessing the reproductive success of *A. crenata* as a potential biomarker of stress. In addition, I acknowledge the limitation of this survey as reproductive sampling effort of the current study appears to be a snapshot one-off occurrence and at any one time there maybe differences in peak spawning of subpopulations of *A. crenata* inhabited across the Estuary, further complicating the assessment of reproductive success as a biomarker in *A. crenata.*

4.4.6 Relationship of sediment grain size to *A. crenata* metal bioaccumulation and biomarker responses

All sediment-associated trace metals (As, Cd, Cu, Ni, Pb and Zn) tested in the present study showed a significant positive correlation with the finest size fraction (< 63 μm) of the sediment. The concentration of Cu, Zn and Cd in *A. crenata* tissues also exhibited a significant positive trend with the sediment finest fraction (< 63 μm). This indicates that the Cu, Zn and Cd associated with finer sediments could be a major uptake route of those metals in these gastropod species. This is in accordance with the findings of Gundacker, (2000) who showed for gastropods, *Radix ovata* and *Viviparus sp.* that sediment-associated Cu, Zn and Cd were more bioavailable to mollusc species. In contrast, inconsistencies between sediment grain size and concentration of Ni, As and Pb in snail tissues may be attributed to either the lack of bioavailability of those metals in the sediment, e.g. due to high adsorption capacity, presence of metal binding agents and metal speciation (Gundacker, 2000) or availability of other sources of metal uptake routes (e.g. direct diffusion of free ions in water through the foot).

The observed relationship between sediment grain size and *A. crenata* CI (for sand, > 125 μm; $R = 0.72$, $p < 0.05$) and GST activity (for clay/silt, < 63 μm; $R = 0.84$, $p < 0.05$) indicated that these gastropod species may be exposed to more environmental toxicity when they are closely associated with a finer sediment. This is because finer particles can trap more contaminants due to high sorption capacity and ultimately accumulate in soft tissues of these deposit feeding organisms via ingestion.

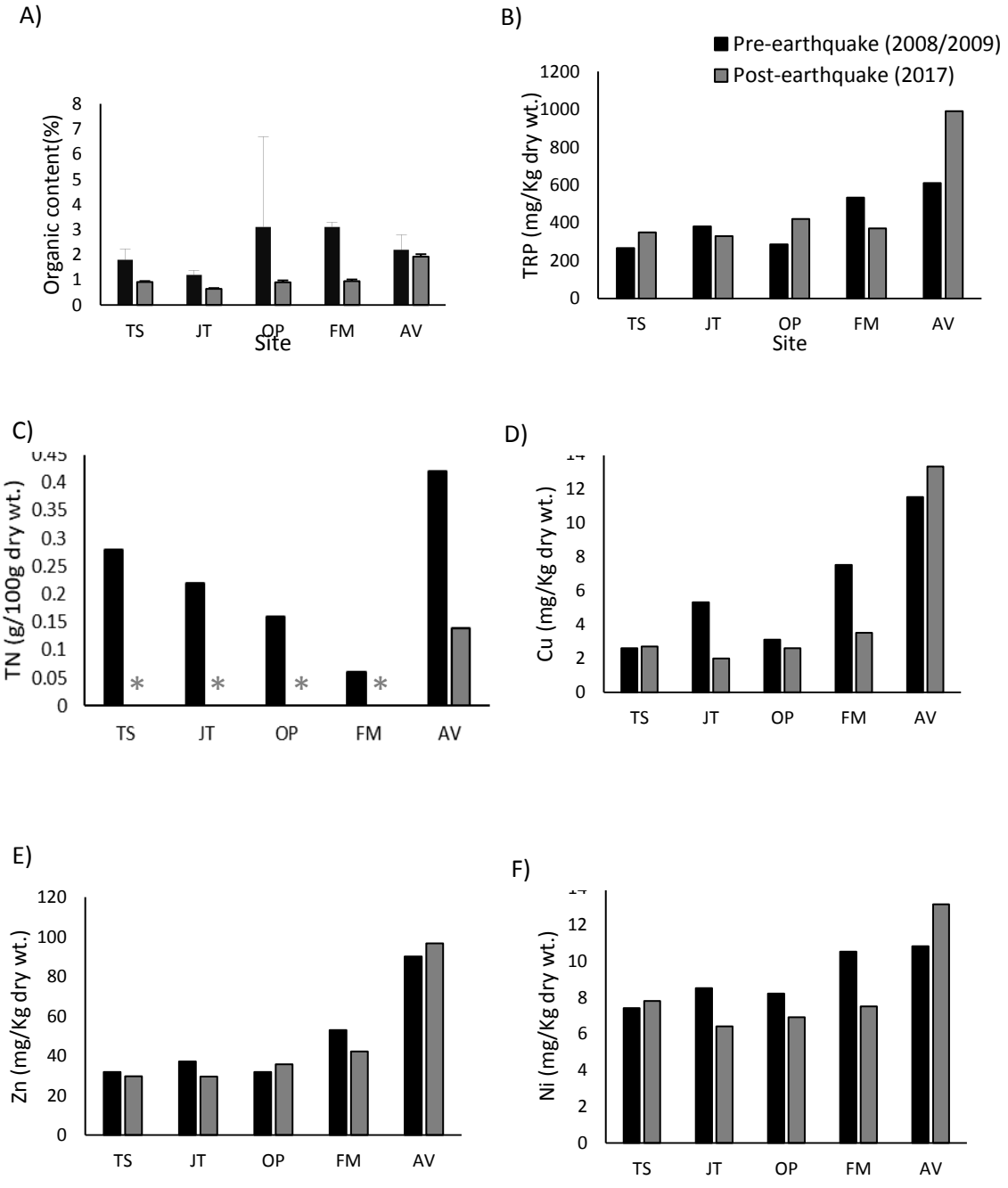
4.4.7 Post-earthquake evaluation of sediment properties, *A. crenata* population density, and condition index

Between 2010-2012, Canterbury, New Zealand, experienced a number of high magnitude earthquakes as a result of the movement of the Australian and Pacific tectonic plates (Skilton, 2013). Among these, the February and June 2011 earthquakes caused considerable damage to the Avon-Heathcote Estuary, including widespread liquefaction, lifting of the southern part of the Estuary (Estuary mouth and Heathcote River mouth) by 0.3-0.5 m, subsidence of the northern part of the Estuary (Avon River mouth) by 0.2-0.5 m and increased area exposed at mid-tide by an average of 18% (Cochran et al., 2014). As a result, the Estuary has undergone significant morphological, sedimentological and biological alterations. Liquefaction caused exchange old sediment with new relatively pristine sediment (approx. 20 – 40 % of the Estuarine bed; Measures, 2011), and along with diversion of the waste outfall directly to the ocean, this resulted in a reduction of trace metals, nutrients and organic content in the Estuarine sediment (Skilton, 2013). In fact, all of these disturbances considerably affected the distribution pattern and population structure of the benthic communities within the Estuary.

The pre- and post-earthquake conditions of sediment properties, *A. crenata* density and condition index are presented in Table 4.7 and Fig. 4.8. After the major Earthquake events in 2010 – 2012 and diversion of sewage outfall, the sediment organic content of the Avon-Heathcote Estuary showed an overall reduction of 52% compared to pre-earthquake measurements. Sediment organic

content levels measured in all five sites (TS, JT, OP, FM and AV) in the present study (ranging from 0.6 – 1.9%) were within the "good organic condition" ratings (< 2% of organic content) developed for estuaries in Southland, New Zealand (Robertson, 2006); However, the organic content of pre-earthquake sediments was more variable (ranging from 1.2 – 3.1%) with three sites (OP, FM and AV) exceeding the thresholds for "good organic condition" ratings, reflecting that the estuarine sediment organic conditions were improved following the Earthquake disturbances and outfall diversion. *D*ensity distribution of *A. crenata* and condition index of adult individuals of all five sites (except density of OP site) showed relatively high values when compared to the pre-earthquake data with an overall increase of 38% and 100% respectively (Fig. 4.8; Table 4.7). This could be attributed to environmental changes following both the outfall diversion and earthquake disturbances i.e. reduction in sediment contaminants due to the introduction of large quantities of new sediments from deeper layers that capped the surface (Measures, 2011; Skilton 2013).

The improved organic conditions in the post-earthquake sediments resulted in increased growth of benthic microalgae biomass (Skilton, 2013) in the Estuary. This may explain the enhanced population growth and condition index of the mud snails since benthic microalgae are the dominant food source of this species (McClatchie et al., 1982; Juniper, 1987). Overall, the results of the present study indicate that post-earthquake condition of the Avon-Heathcote Estuary has been improved due to physicochemical changes associated with both earthquake disturbances and outfall diversion and suggests that current conditions are more favourable for the intertidal benthic communities.


A)
Organic content(%)
0
1
2
3
4
5
6
7
8
TS
JT
OP
FM
AV
Site
B)
Pre-earthquake (2008/2009)
Post-earthquake (2017)
TRP (mg/Kg dry wt.)
0
200
400
600
800
1000
1200
TS
JT
OP
FM
AV
Site
C)
TN (g/100g dry wt.)
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
*
*
*
*
TS
JT
OP
FM
AV
D)
Cu (mg/Kg dry wt.)
0
2
4
6
8
10
12
TS
JT
OP
FM
AV
E)
Zn (mg/Kg dry wt.)
0
20
40
60
80
100
120
TS
JT
OP
FM
AV
F)
Ni (mg/Kg dry wt.)
0
2
4
6
8
10
12
TS
JT
OP
FM
AV

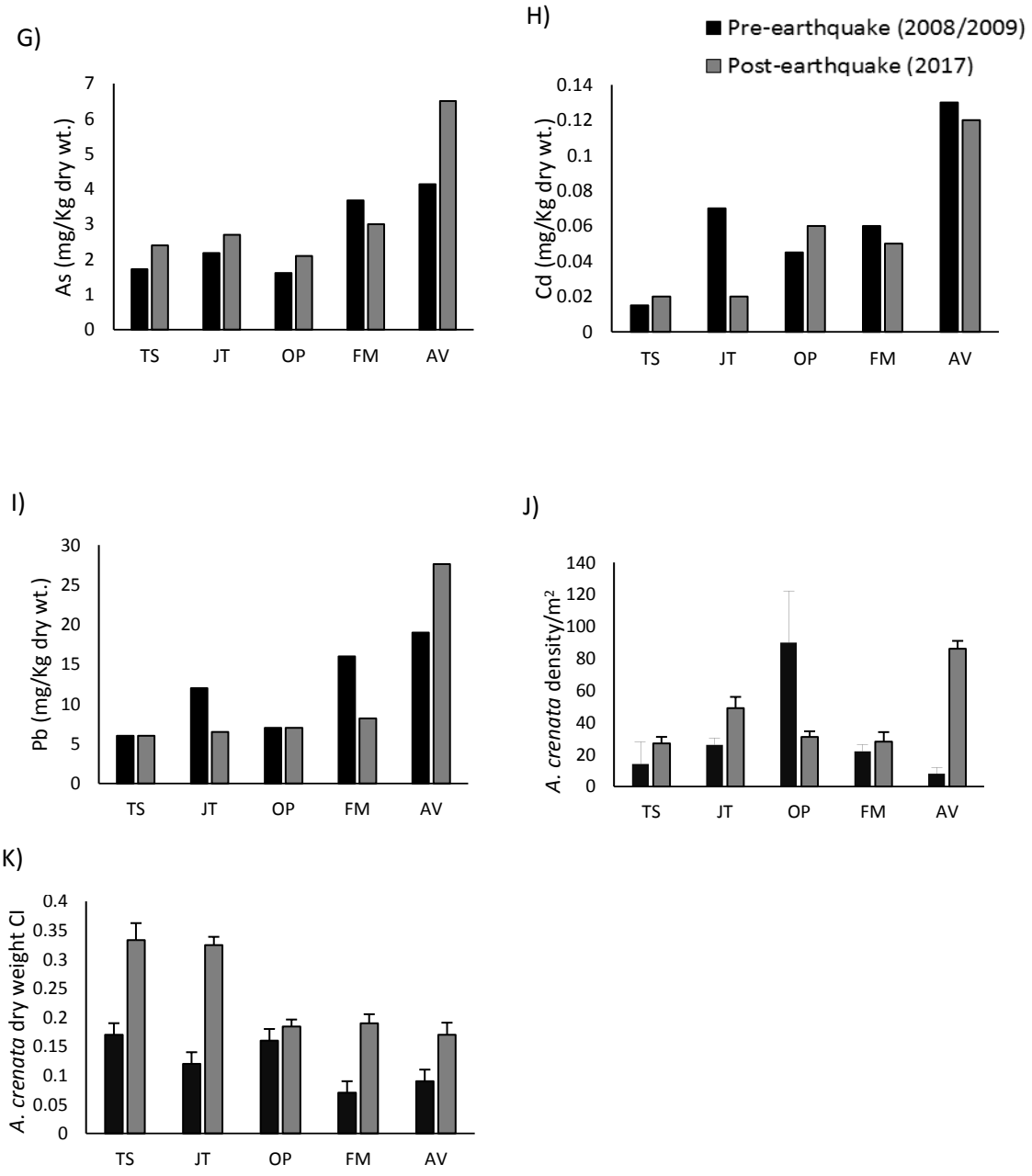


Figure 4.8 Comparison of pre- (2008/2009) and post-earthquake (2017) environmental parameters and *A. crenata* characteristics of the Avon-Heathcote Estuary. Organic content (A); total recoverable phosphorous (TRP) (B); total nitrogen (TN) (C); Cu (D); Zn (E); Ni (F); As (G); Cd (H); Pb (I); in the sediment and *A. crenata* density (J); condition index (K). TS, Tern Street; JT, Pleasant Jetty; OP, Oxidation ponds; FM, Ferrymead and AV, Avon.

* TN < 0.05 g/100g

Table 4.7 Comparison of sediment properties, *A. crenata* density, and condition index in the Avon-Heathcote Estuary before and after the 2010 – 2012 earthquake events. Data are presented as mean ± SEM over the five sites of Tern Street (TS), Pleasant Jetty (JT), Oxidation ponds (OP), Ferrymead (FM) and Avon (AV).

Parameter	Pre-earthquake 2008/2009	Post-earthquake 2017	Change
Sediment			
Organic matter	2.3 ± 0.3	1.1 ± 0.2	52%▼
TRP	415 ± 60	492 ± 112	18%▲
TN	0.23 ± 0.05	< 0.05 - 0.14	min. of 39%▲
Cu	6.0 ± 1.5	4.8 ± 1.9	20%▼
Zn	56.2 ± 9.6	46.7 ± 11	17%▼
Ni	9.1 ± 0.6	8.3 ± 1.1	9%▼
As	2.7 ± 0.5	3.3 ± 0.7	22%▲
Cd	0.06 ± 0.02	0.05 ± 0.02	17%▼
Pb	12.0 ± 2.2	11.1 ± 3.7	7.50%▼
A. crenata			
Density/m²	32 ± 13	44 ± 10	38%▲
Condition index	0.1 ± 0.02	0.2 ± 0.03	100%▲

4.5 CONCLUSIONS

In conclusion, the sites in the Avon-Heathcote Estuary were distinguishable according to the metal content of the mud snail tissues, metals, nutrients and organic matter content in the sediment, and the biomarker responses. Metal accumulation in *A. crenata* reflects the environmental levels, suggesting that tissue metal concentration of this species is cable of being used as an indicator of environmental exposure. Previous studies have shown that *A. crenata* is tolerant to benthic and aquatic contaminants and population attributes such as growth, survival and density of this species could be useful as an ecological bioindicators for monitoring estuarine condition. The findings of the present study also supported the concept that the population attributes of *A. crenata* including length structure and density are useful as a monitoring tool for environmental stressors including both anthropogenic and natural disturbances in estuaries. In addition, the results of the present study confirmed the usefulness of CI and GST activity as biomarkers of exposure to contaminants in this mud snail. While acknowledging the limitation of the study that the reproductive success

survey was a one-off occurrence, the reproductive success of *A. crenata* appears to be dependent on a number of site-specific biotic and abiotic factors, and thus complicate the task of assessing the reproductive success of this species as a biomarker of stress in their natural environments. Earthquake disturbances and outfall diversion had a positive impact on the Avon-Heathcote Estuary environmental conditions and it is likely that new conditions are more favourable for the intertidal benthic communities of the Estuary.

Chapter 5

Attributes of *Amphibola crenata* population attributes as indicators of coastal contaminants

5.1 INTRODUCTION

Populations of many aquatic invertebrates are affected by a variety of stressors caused by human activities and exhibit considerable variability in space and time under the influence of those stressors (Crowe et al., 2000). For example, Gappa et al., (1990) reported increased density of the intertidal, pulmonate limpet, *Siphonaria lesson* and decreased density of the mussel, *Brachidontes rodriguezi* in areas highly polluted with sewage outfall. Nagelkerken and Debrot, (1995) also found reduced density and diversity of molluscs communities in coastal areas contaminated with oil wastes. In another study, Castilla, (1996) found that barnacle communities were replaced by copper-resistant algae in coastal areas contaminated by copper mining residuals.

Aquatic gastropods are exposed to numerous natural and anthropogenic contaminants from industrial waste, agricultural runoff, and municipal wastewater discharges (Cardoso et al., 2013; Marsden and Swinscoe, 2014). Of all contaminants, trace metals and nutrients are considered to be the most common contaminants to which aquatic organisms are exposed. Estuaries often exhibit high levels of trace metals and nutrients because of the elevated use of estuarine catchment areas for commercial, industrial, and agricultural purposes worldwide (e.g. Pan and Wang, 2012; Marsden et al., 2014). The direct or indirect effects of trace metals and nutrients on organisms can eventually result in stress responses ranging from the biochemical/physiological to the population and community level changes (Lagadic et al, 1994; Marsden and Swinscoe, 2014; Zhang et al., 2014).

Intertidal gastropods are good indicators of estuarine contaminants (Clarke and Ward, 1994; Cardoso et al, 2013; Marsden and Baharuddin, 2014). They exhibit a sedentary lifecycle, have a close association with sediment and a long-life span that integrates contaminant impact over time (Mucha et al., 2003). Biomarker responses in aquatic invertebrates have been increasingly used as indicators of environmental changes. Population level biomarker responses are widely evaluated as indicators for variety of environmental stressors (Eschweiler et al., 2009; Cardoso et al., 2013). Some attributes include length group, population density, sex ratio, and growth rates (Shriver et al., 2002; Marsden and Swinscoe, 2014).

Population attributes of the intertidal pulmonate gastropod, *Amphibola crenata,* have been evaluated as potential indicators for trace metal and nutrient contaminants for NZ estuarine

habitats. Population abundance, model length group, growth rate, and condition index of *A. crenata* have been investigated in relation to estuarine contaminants (Marsden and Swinscoe, 2014; Marsden and Baharrudin, 2014). Nevertheless, these studies were confined to a single estuary. The current study is the first to investigate population attributes (e.g. density and length distribution) of *A. crenata* on a regional scale to assess their use as trace metal and nutrient indicators for NZ estuaries. Because such population level measurements can be less expensive than chemical analyses, they might be useful as management tool for evaluating the contaminants within coastal ecosystems. Therefore, the main objective of the present study was to assess *A. crenata* population attributes as indicators of sediment trace metal and nutrient pollution.

5.2 METHODS

5.2.1 Study area

The study was undertaken in 17 estuarine sites in six different regions including Auckland, Canterbury, Nelson, Southland, Waikato and West Coast. Environmental parameters including salinity, temperature, the depth of the anoxic layer down to 15 cm from the surface, and sediment characteristics were measured for each of the site (Table 5.1). All of the sites used for the population structure study in the present study were identical to the sites that were used to collect snail samples for biomarker studies and site locations are provided in Chapter 6 (Fig. 6.1).

5.2.2 Distributional survey

The population structure (e.g. density and/or length distribution) of *Amphibola crenata* was assessed at mid-level at 17 locations. Sampling of South Island sites (Canterbury, Nelson, Southland, and West Coast) were conducted in January and Feb 2016, while the North Island (Auckland and Waikato) sampling was undertaken in Feb and March 2017. At each site (except for New River, Karamea and Firth of Themes), a 30 x 15 m plot was marked and subdivided it into four 15 x 7.5 m plots (Fig. 5.1). Within each subplot, five random quadrats of 0.5 x 0.5 m were placed, and the shell length and density of all live snails within the quadrat were recorded. The GPS positions of the temporary plots were recorded at each site. Mud snails in New River and Jacobs River in Southland and Karamea in West coast showed a highly patchy distribution, and

were excluded from density estimations, and only snail length was recorded for a minimum of 60 individuals which were collected randomly.

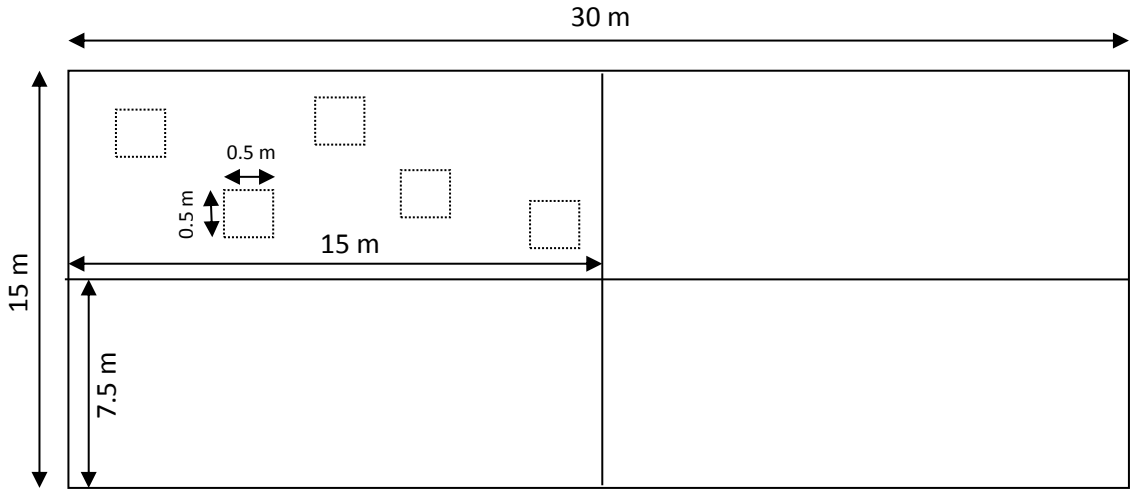


Figure 5.1 Experimental design used to measure population structure of *A. crenata.*

Table 5.1 Habitat characteristics and environmental parameters of the sampling sites. Depth of anoxic layer was measured only if it appears within the top 15 cm from the surface.

Region	Sample site	Location	Potential sources of contaminants	Sediment type	Anoxic layer depth (cm)	Water temperature (°C)	Salinity (‰)	pH	Proximity to open sea (Km)
Canterbury	Ashley River	S 43° 16. 843' E 172° 43.185'	Agricultural runoff	Fine sand	11.0	22.7	7.4	7.3	1.5
	Avon-Heathcote	S 43° 33. 384' E 172° 42.413'	Industrial discharge	Fine sand, very fine sand	-	13.5	23.4	6.8	4.6
	Charteris Bay	S 43° 39. 337' E 172° 41.913'	Influenced by Governors Bay sewage treatment discharge and agricultural discharge	Clay, Silt	0.5	20.9	32.2	8.5	4.0
Nelson	Ligar Bay	S 40° 49. 008' E 172° 54.875'	Domestic discharge	Fine sand	1.5	24.9	33.8	8.4	0.8
	Parapara Inlet	S 40° 43. 905' E 172° 41.061'	Agricultural runoff, domestic discharge	Silt, fine sand	-	22.2	25.5	7.7	1.9
	Mapua	S 41° 15. 611' E 173° 04.668'	City and industrial effluents, historically contaminated by pesticides	Clay, silt	-	21.2	31.9	7.7	4.0
Southland	Waikawa	S 46° 36. 479' E 169° 08.121'	Agricultural runoff	Clay, silt	4.0	24.0	26.9	8.2	4.0
	Jacobs River	S 46° 20. 111' E 167° 58.013'	Urban and agricultural stormwater runoff, industrial discharge	Clay, silt	0.5	14.7	15.8	7.7	5.9
	New River	S 46° 25. 588' E 169° 19.483'	Urban and agricultural stormwater runoff, industrial discharge	Clay, silt	-	17.1	11.6	7.5	12.1
West Coast	Karamea	S 41° 16. 138' E 172° 06.401'	Agricultural runoff	Fine sand, very fine sand	20.0	17.2	16.3	5.9	2.2
	Orowaiti	S 41° 44. 821' E 171° 36.796'	Leaching from landfill	Clay, Silt	0.5	16.3	10.2	5.4	5.3
Waikato	Taiuru Estuary	S 36° 59. 728' E 175° 51.458'	Recreational activity	Medium sand	1.0	25.5	27.8	7.2	1.5
	Waikato River Estuary	S 37° 23. 301' E 174° 43.634'	Urban and industrial waste	Fine sand	0.5	23.2	21.6	5.8	8.5
	Firth of Thames	S 37° 10. 917' E 175° 19.434'	City and industrial discharge	Clay, silt, very fine sand	3.0	27.0	23.4	5.1	2.2
Auckland	Papakura Inlet	S 37° 04. 280' E 174° 54.297'	City and industrial discharge	Clay, Silt	-	17.8	29.2	7.2	7.1
	Waiuku Estuary	S 37° 11. 757' E 174° 42.248'	Industrial discharge	Fine sand	-	19.6	30.4	6.9	13.2
	Orewa Estuary	S 36° 35. 783' E 174° 40.902'	Urban and agricultural runoff	Clay, Silt, very fine sand	-	27.3	30.9	6.7	2.1

5.2.3 Sediment trace metals and nutrients analysis

Sediment samples (n = 8) were collected within each plot (these sediment samples were also used in the physiological and biochemical biomarker assessment) and analysed for trace metals of arsenic (As), cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb) and zinc (Zn). Detailed protocols for the sediment trace metals analysis are provided in Chapter 4.

A single sediment sample from the surface layer (to a ~ 2cm depth) was taken from each plot at each site. Sediment samples were analysed for total recoverable phosphorous (TRP) and total nitrogen (TN) at Hill Laboratories. TRP was analysed using nitric/hydrochloric acid digestion, ICP-MS, screen level (US EPA 200.2). Detection limit for TRP was 40 mg kg^{-1} dry wt. TN was analysed using catalytic combustion, separation and thermal conductivity and detection limit for TN was 0.05 g/100 g dry wt.

5.2.4 Sediment organic content and particle size distribution

Sediment organic content (OC) and particle size distribution were measured at each of the site and detailed protocols are provided in Chapter 4 (sections 4.2.5 & 4.2.6)

5.2.5 Data analysis

5.2.5a Descriptive statistics

Shell length variables of *A. crenata* including minimum, maximum, mean and median values were calculated for each of the site to assess site-specific relationships of those variables. In order to assess relationships of *A. crenata* population attributes to sediment trace metals and nutrients, snails were divided into three size classes including small (< 7.9 mm), medium (7.9 – 18 mm) and large (> 18 mm).

5.2.5b Statistical Analyses

Data were processed using R statistical software (R version 3.0.2). Trace metal in the sediment and snail length distribution were compared between sites using a one-way nested ANOVA

followed by a post-hoc test (TukeyHSD). All data were tested for, and passed, normality and homogeneity of variance assessments using Shapiro-Wilk and Flinger-Killeen tests before being analysed. The Spearman rank correlation (R) was conducted to determine the relationship of *A. crenata* densities to sediment trace metals and TRP. TN values were excluded from the correlation analysis due to low detection limits for some sites. A value of $p < 0.05$ was considered statistically significant.

5.3 RESULTS

5.3.1 Sediment trace metals and nutrients

With the exception of Ni concentration in the Mapua, Parapara Inlet and New River, and As concentration in the Waiuku, surface sediment content of sites exhibited relatively low levels of metals, zinc (0.9 – 148. 3 mg kg^{-1}), copper (0.1 – 24.9 mg kg^{-1}), Ni (0.2 – 13.0 mg kg^{-1}), As (0.1 – 21.7 mg kg^{-1}), Cd (0.001 – 0.143 mg kg^{-1}) and Pb (0.2 – 23.5 mg kg^{-1}). Sediment trace metals however, showed significant regional and site-specific variations (Table 5.2). Sediment TRP of the sites were ranged from 220 – 1960 mg kg^{-1}, while sediment TN ranged < 0.05 – 0.56 mg kg^{-1}. In 8 out of 17 sites, sediment nitrogen levels were less than the detection limit of 0.05 mg kg^{-1}.

5.3.2 Sediment organic content and particle size distribution

Organic content of the study sites varied from 0.1 – 0.9 g kg^{-1}. The highest sediment organic content was reported at Orewa site in the Auckland, while the lowest organic content was reported at Karamea in the West Coast. Organic content showed a significant negative correlation to sediment fine sand fraction ($R = 0.52$, $p < 0.05$; Table 5.5). Sediment particle size showed a wide range of variation, whereas the Orewa site recorded the higher percentage of silt/clay (Fig. 5.2).

Table 5.2 Trace metals (expressed as mg kg^{-1}; n = 8), total recoverable phosphorus (TRP; n = 1), total nitrogen (TN; n = 1) and organic content (OC) concentrations of sediment samples collected from 17 sites in NZ. For trace metals, data are presented as mean ± SEM. Upper case letters indicate significant differences between regions, while lower case letters displayed significant differences between sites within each region as determine by one-way nested ANOVA followed by Tukey post-hoc test at $p < 0.05$. Significant differences were based on sediment trace metal concentrations. Graphical representation of sediment trace metal distribution is provided in Chapter 6; Fig. 6.2.

Region	Site	Metal						TRP (g/kg)	TN (g/100g)	OC (g/kg)
		Cu	Zn	Ni	As	Cd	Pb			
Canterbury	Ashley River	4.1 ± 0.4^{a}	34.7 ± 1.1^{a}	9.4 ± 0.3^{a}	3.6 ± 1.5^{a}	0.009 ± 0.001^{a}	7.8 ± 0.2^{a}	440	<0.05	0.55
(CA)	Avon-Heathcote	2.9 ± 0.2^{a}	54.6 ± 2.2^{b}	7.2 ± 0.2^{b}	3.0 ± 1.2^{a}	0.079 ± 0.005^{b}	9.3 ± 0.4^{b}	340	<0.05	0.42
	Charteris Bay	5.5 ± 0.2^{c}	63.2 ± 2.2^{c}	13.0 ± 0.4^{c}	6.1 ± 2.5^{b}	0.019 ± 0.001^{a}	17.8 ± 0.3^{c}	830	0.1	0.89
		AB	A	AC	A	AD	A			
Nelson	Ligar Bay	5.8 ± 1.1^{a}	25.9 ± 4.7^{a}	6.7 ± 1.1^{a}	3.8 ± 1.5^{a}	0.004 ± 0.001^{a}	2.9 ± 0.5^{a}	620	0.1	1.12
(NE)	Parapara Inlet	10.0 ± 0.4^{b}	40.4 ± 1.9^{b}	28.3 ± 1.0^{b}	8.5 ± 3.5^{b}	0.015 ± 0.002^{b}	5.0 ± 0.2^{b}	460	<0.05	0.84
	Mapua	5.7 ± 0.1^{a}	32.5 ± 0.9^{ab}	59.0 ± 1.5^{c}	7.8 ± 3.2^{b}	0.006 ± 0.001^{a}	9.3 ± 0.3^{c}	619	<0.05	0.68
		AC	A	B	A	AC	B			
Southland	Waikawa	4.0 ± 0.2^{a}	38.2 ± 1.5^{a}	5.2 ± 0.2^{a}	4.9 ± 2.0^{a}	0.017 ± 0.001^{a}	2.9 ± 0.2^{a}	510	0.1	0.92
(SO)	Jacobs River	19.5 ± 3.3^{b}	43.6 ± 5.9^{a}	10.1 ± 1.3^{b}	6.4 ± 2.6^{b}	0.049 ± 0.009^{b}	3.8 ± 0.6^{a}	710	0.2	0.22
	New River	24.9 ± 0.5^{b}	148.3 ± 2.7^{b}	28.9 ± 0.7^{c}	8.9 ± 3.6^{c}	0.143 ± 0.003^{c}	15.7 ± 0.3^{b}	1830	0.3	0.39
		D	C	A	A	B	A			
West Coast	Karamea	0.1 ± 0.1^{a}	0.9 ± 0.1^{a}	0.2 ± 0.1^{a}	0.1 ± 0.01^{a}	0.001 ± 0.0001^{a}	0.2 ± 0.01^{a}	570	<0.05	0.10
(WC)	Orowaiti	0.4 ± 0.1^{b}	1.9 ± 0.2^{b}	0.5 ± 0.1^{b}	0.3 ± 0.01^{b}	0.001 ± 0.0001^{b}	0.4 ± 0.01^{a}	1250	0.1	0.23
		B	B	C	B	C	C			
Waikato	Taiuru	4.5 ± 0.1^{a}	31.1 ± 2.6^{a}	4.7 ± 0.3^{a}	15.1 ± 0.5^{a}	0.019 ± 0.01^{a}	5.9 ± 1.7^{a}	220	<0.05	0.46
(WA)	Waikato River	5.7 ± 0.3^{b}	54.1 ± 1.2^{b}	7.6 ± 0.2^{b}	14.6 ± 0.6a	0.024 ± 0.001^{a}	3.8 ± 0.3^{b}	520	<0.05	0.30
	Firth of Thames	5.9 ± 0.1^{b}	64.7 ± 1.1^{c}	5.6 ± 0.1^{a}	4.5 ± 0.1^{b}	0.074 ± 0.003^{b}	23.5 ± 0.3^{c}	680	0.2	0.86
		A	A	AC	C	D	A			
Auckland	Papakura Inlet	5.1 ± 1.0^{a}	52.9 ± 1.2^{a}	5.6 ± 0.3^{a}	15.2 ± 1.4^{a}	0.036 ± 0.001^{a}	13.2 ± 0.3^{a}	1010	0.2	0.68
(AU)	Orewa	7.7 ± 0.2^{a}	50.9 ± 1.5^{a}	5.6 ± 0.2^{a}	16.1 ± 1.7^{a}	0.037 ± 0.002^{a}	6.5 ± 0.4^{b}	1960	0.6	0.91
	Waiuku	22.1 ± 0.4^{c}	62.4 ± 1.9^{b}	6.9 ± 0.3^{a}	21.7 ± 0.3^{b}	0.015 ± 0.004^{b}	6.8 ± 0.2^{b}	340	<0.05	0.35
		CD	AC	AC	D	AD	A			

5.3.3 Abundance of *Amphibola crenata*

Density of *A. crenata* varied widely among sites (Table 5.3). The highest densities were recorded at Orewa (72 ind/m^2) and the lowest densities were found at Taiuru (8 ind/m^2) and Waikato River (8 ind/m^2). The Mapua, Orowaiti, Papakura Inlet, Orewa and Waiku sites were dominated by medium (7.9 – 18 mm) individuals, while all other sites were dominated by large (> 18 mm) individuals. Relatively low abundance of small (< 7.9 mm) individuals were recorded for all sites. Densities of large individuals showed a significant negative correlation to sediment As concentration ($R = -0.57$; $p < 0.05$), while density of medium individuals showed a significant positive correlation to sediment phosphorus concentration ($R = 0.9$; $p < 0.001$;) and negative correlation to sediment fine sand fraction ($R = 0.54$; $p < 0.05$; Table 5.5).

Table 5.3 Density of *Amphibola crenata* (ind/m^2). Densities were not available for 3 sites, Jacobs River, New River and Karamea sites due to highly patchy distribution. For total snail density, data are presented as mean ± SEM.

Sites	ind/m^2			
	Small (< 7.9 mm)	Medium (7.9 – 18 mm)	Large (> 18 mm)	Total
Ashley River	11	7	33	51 ± 3
Avon-Heathcote	0	1	42	43 ± 2
Charteris Bay	1	20	30	51 ± 6
Ligar Bay	2	4	15	21 ± 2
Parapara Inlet	0	0.5	13	13.5 ± 2
Mapua	1	17	6	24 ± 5
Waikawa	3	11	33	47
Jacobs River	-	-	-	-
New River	-	-	-	-
Karamea	-	-	-	-
Orowaiti	4	38	23	65 ± 6
Taiuru	0	1	7	8 ± 1
Waikato River	0	1	7	8 ± 1
Firth of Thames	1	12	16	29 ± 1
Papakura Inlet	0	33	25	58 ± 8
Orewa	0	50	22	72 ± 10
Waiuku	4	12	8	24 ± 6

5.3.4 Population structure

*P*opulation structure of *Amphibola crenata* showed a unimodal or bimodal distribution (Fig. 5.3) and also exhibited a significant regional (ANOVA $F = 81.7$, $p < 0.01$) and site-specific variations (ANOVA $F = 67.9$, $p < 0.01$; Table 4). Snails from New River site in the Southland Region recorded the highest mean shell length of 31.5 mm and lowest mean shell length of 12.5 mm were found at Orowaiti site in the West Coast Region (Table 5.4). The Mapua, Orowaiti, Papakura Inlet, Orewa and Waiuku sites recorded high proportion of medium (7.9 – 18 mm) individuals, while all other sites recorded high proportion of large (>18 mm) individuals. Only two sites including Ashley River and Waiuku sites recorded proportion of small individuals higher than 10% (Fig. 5.3). Minimum, median and mean shell length of *A. crenata* positively correlated with sediment Cd and Zn concentrations (Table 5.5).

Table 5.4 Descriptive statistics (minimum, maximum, mean ± SD and median) of *A. crenata* length distribution among the sampling sites. Upper case letters indicate significant differences between regions, while lower case letters displayed significant differences between sites within each region as determine by one-way nested ANOVA followed by Tukey post-hoc test at $p < 0.05$.

Region	Sites	Min	Max	Mean	Median
Canterbury^{AB}	Ashley River^{b}	4.4	33.8	18.9 ± 8.2	23.3
	Avon-Heathcote^{a}	16.2	25.8	21.9 ± 1.5	21.6
	Charteris Bay^{b}	6.7	23.5	17.8 ± 3.6	19
Nelson^{A}	Ligar Bay^{a}	5.4	23.5	18.9 ± 4.3	20.4
	Parapara Inlet^{b}	14.8	23.7	20.4 ± 1.5	20.5
	Mapua^{c}	5.3	23	15.5 ± 3.6	16.3
Southland^{C}	Waikawa^{a}	5.6	28.4	20.9 ± 6.3	23.8
	Jacobs River^{a}	8.5	30.5	21.8 ± 3.7	22.6
	New River^{b}	22	35.6	31.5 ± 2.4	32
West Coast^{D}	Karamea^{a}	7.1	29.2	18.9 ± 4.9	18.6
	Orowaiti^{b}	4.2	21.2	12.5 ± 2.9	12.8
Waikato^{B}	Taiuru^{a}	9.5	26	21.4 ± 3.1	22.3
	Waikato River^{b}	11.2	23.3	19.9 ± 1.8	20.0
	Firth of Thames^{c}	6.7	23.6	17.5 ± 3.0	18.2
Auckland^{D}	Papakura Inlet^{a}	8	23.5	17.2 ± 2.8	17.4
	Orewa^{a}	11.2	23.2	17.1 ± 3.0	16.4
	Waiuku^{b}	3.2	23.3	14.8 ± 5.6	16.3

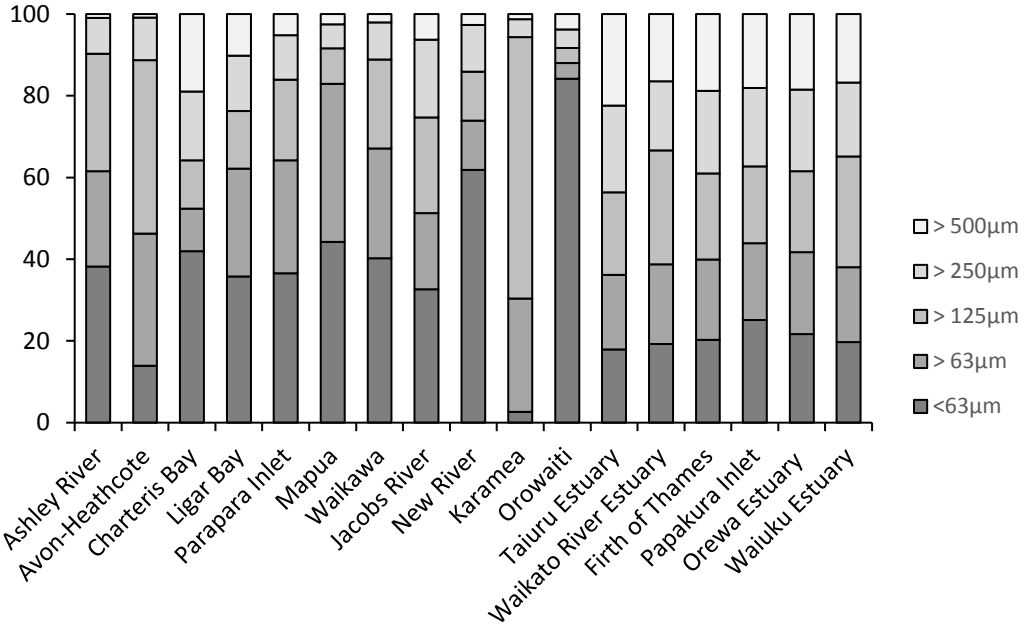


Figure 5.2 Particle size distribution of sediments in sampling sites (by %). The nominal size range of sediments were: coarse sand > 500 µm; medium sand > 250 µm; fine sand > 125 µm; very fine sand > 63 µm and clay/silt < 63 µm.

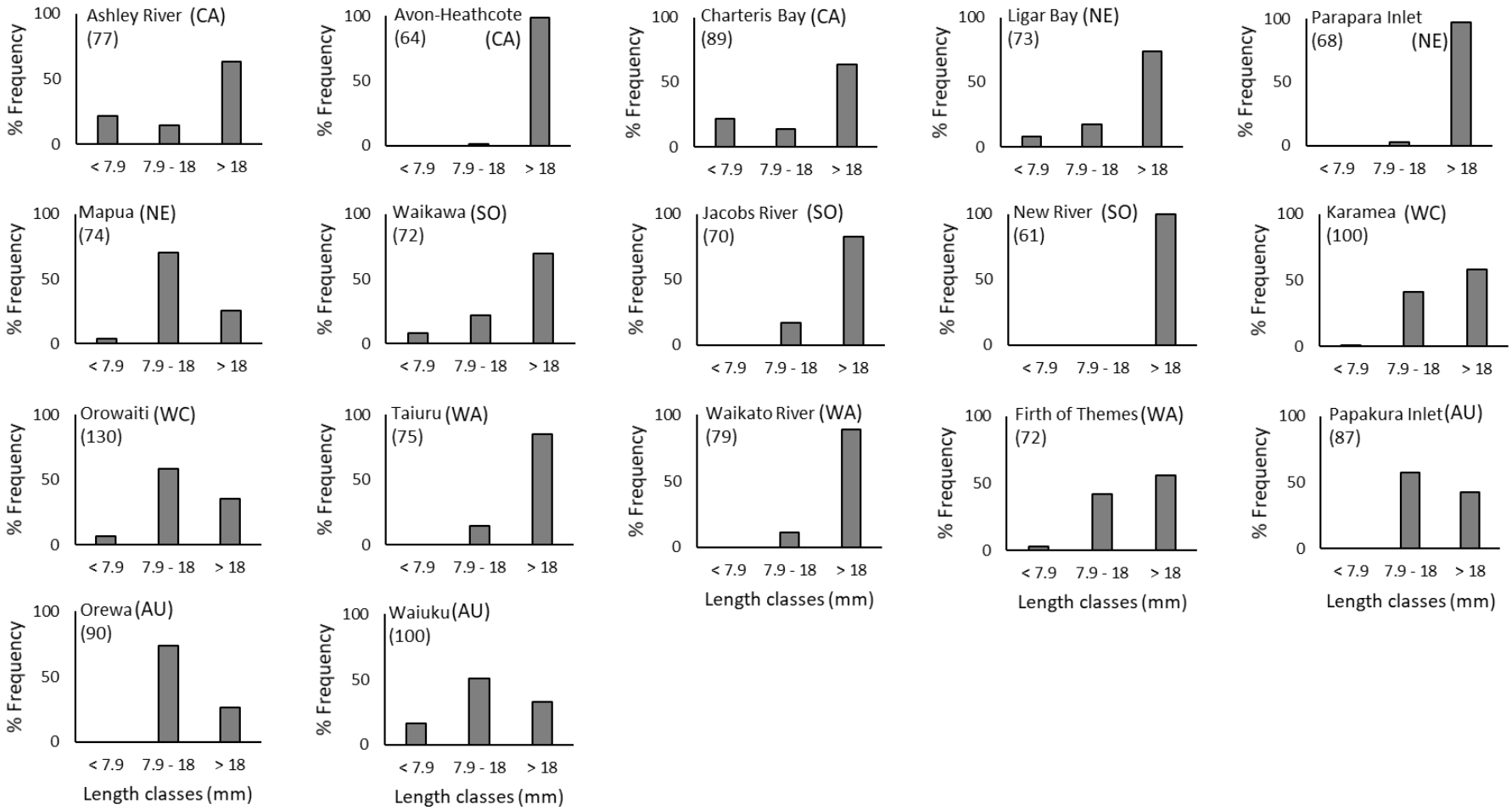


Figure 5.3 Size distribution of (% frequency) *A. crenata* in different areas. Sample number was shown in brackets, CA – Canterbury, NE – Nelson, SO – Southland, WC – West Coast, WA – Waikato and AU – Auckland.

Table 5.5 Correlation (R) analysis of *A. crenata* population attributes with sediment characteristics. TRP – total recoverable phosphorus, OC – organic content, C/S – clay/Silt fraction, F/sand – fine sand fraction. Values with bolt indicate significant differences determined by Spearman rank correlation. A value of $p < 0.05$ was considered statistically significant.

	Sediment										*A. crenata*						
	Cu	Zn	Ni	As	Cd	Pb	TRP	OC	C/S	F/sand	Small	Medium	Large	Min	Max	Mean	Median
Cu																	
Zn	0.71																
Ni	0.26	0.27															
As	0.45	0.33	0.03														
Cd	0.54	0.86	0.12	0.04													
Pb	0.2	0.64	0.21	0.09	0.58												
TRP	0.26	0.44	0.06	0.08	0.45	0.2											
OC	-0.17	0.06	0.16	0.02	-0.1	0.33	0.09										
C/S	0.13	0.13	0.29	-0.31	0.07	-0.02	0.43	0.35									
F/sand	-0.2	-0.24	-0.37	-0.16	-0.06	-0.26	-0.38	**-0.52**	-0.74								
Small	0.03	-0.29	-0.16	-0.25	-0.37	-0.19	-0.02	-0.18	0.35	0.06							
Medium	-0.2	-0.25	-0.06	-0.12	-0.19	0.14	**0.9**	0.07	0.36	**-0.54**	0.01						
Large	-0.45	0.06	-0.35	**-0.57**	0.35	0.13	0.16	0.11	0.12	0.34	0.15	-0.24					
Min	0.37	**0.66**	0.23	0.08	**0.77**	0.21	0.37	-0.11	0	0.06	**-0.59**	-0.22	0.15				
Max	0.38	0.4	0.04	-0.24	0.46	0.03	0.1	-0.31	0.06	0.28	0.7	-0.37	0.47	0.36			
Mean	0.48	**0.69**	0.15	-0.04	**0.74**	0.18	0.19	-0.1	0.01	0.11	-0.22	**-0.66**	0.24	**0.8**	**0.74**		
Median	0.46	**0.64**	0.14	-0.09	**0.64**	0.17	0.07	-0.04	0.06	0.06	0.17	**-0.71**	0.31	**0.63**	**0.83**	**0.96**	

5.4 DISCUSSION

This study found striking differences in the population structure of *A. crenata* in relation to sediment contamination. In particular, length structure and density showed positive relationships with sediment trace metals and nutrients, suggesting that this species may develop greater tolerance to withstand under high contamination levels.

Due to the large dilution effect of tidal flushing in most New Zealand estuaries, studies of surface waters are often not particularly useful as indicators of estuarine condition (Updegraff et al. 1977). Benthic characteristics including both sediment properties and benthic community structure however, are more stable (Turner et al. 1996) and are generally considered to be better integrators of condition in well-flushed estuaries that may have been periodically exposed to contaminants over a long period of time (Roper et al. 1988; Gillespie & MacKenzie 1990,).

Populations of *A. crenata* are widespread throughout New Zealand and have been recorded in most of the estuarine intertidal habitats. It is a dominant member of the intertidal macrofauna (Jones & Marsden, 2005). Previous studies on *A. crenata* population attributes (e.g. density, size distribution, condition index) in relation to the environmental variables (e.g. trace metals, salinity, shore level and nutrients) suggested potential use of this species as a bioindicator for estuarine monitoring in NZ (Bennington, 1979; Marsden and Baharuddin, 2014; Marsden and Swinscoe, 2014). These studies however confined to a few specific sites within single estuary. Therefore, the present study is the first study that evaluated *A. crenata* population attributes as potential indicators of estuarine health using a wide range of geographically different estuarine sites which *A. crenata* inhabited.

The findings of this study demonstrated significant regional and site-specific differences in *A. crenata* populations and abundances. Density of the mud snails was relatively high in sites contaminated with nutrient and organic content (e.g. Charteris Bay, Orowaiti, Papakura Inlet, and Orewa), while highest densities were occurred in the Orewa site, where the highest sediment total recoverable phosphorous (TRP), total nitrogen (TN) and organic content were recorded. In addition, the present study also found a significant positive correlation between medium (7.9 – 18 mm) individuals and sediment TRP, whereas negative correlation was found between medium individuals and sediment fine fraction (> 125 μm). These findings are consistent with previous

studies on the same species (Marsden and Baharuddin, 2014; Chapter 4) and also studies on *Hydrobia ulvae* where higher densities occurred in eutrophicated areas in the Mondego Estuary, Portugal (Cardoso et al., 2002). This high densities of medium-sized *A. crenata* in areas contaminated with nutrients could be attributed to increased food availability, less predatory pressure and less competition from small/large individuals of their own species or other species in contaminated areas (Norkko et al., 2010; Marsden and Swinscoe, 2014). Relationship between sediment particle size and infaunal communities have been studied in a variety of habitat (Ysebaert and Herman, 2002; Coblentz et al., 2015). These studies found that selection of sediment particle size by organisms is highly dependent on the type of organism. For example, Van-Hoey et al. (2004) reported high densities of *Mysella bidentate* in intertidal muddy-fine sand areas and low densities of *Nephtys cirrosa* in well-sorted sandy sediment.

In the present study, a significant negative correlation was found only between densities of large (> 18 mm) individuals and sediment arsenic (As) concentration, while all other sediment trace metals (e.g. Cu, Zn, Ni, Cd and Pb) showed no significant correlation to *A. crenata* density distribution. Sediment Cd and Zn, however, had a significant positive correlation with minimum, median, and maximum length of *A. crenata*. A number of studies have investigated impacts of trace metals and other contaminants on estuarine intertidal communities. Watzin and Roscigno (1997) studied effect of zinc contamination on estuarine benthic invertebrates using both formulated sediment as test substrate and field exposure. These results found that several taxa including polychaetes, copepods and ostracods appeared to be most sensitive to zinc, in contrast, some taxa particularly, gastropods were attracted to zinc-contaminated sediments. Another study by Vinagre et at. (2008) found no effect in abundance of intertidal gastropod, *Hydrobia ulvae* associated with low levels of contamination by Zn, Pb and Cu which is similar to findings of the present study for *A. crenata*. Bennington (1979) also found a high abundance of *A. crenata* in areas with high contamination levels in the Avon-Heathcote Estuary, New Zealand and concluded that this species may have a high resistance to the effects of pollution, compared with other estuarine benthic invertebrates. McGuinness (1990) and Clarke and Ward (1994) studied effects of crude oil residuals on gastropod abundance, and both studies found no evidence of reduced abundance of gastropods at areas previously contaminated with crude oil. Overall, all of these studies,

including the present study, suggest that estuarine gastropods have the ability to survive under a wide range of environmental conditions, particularly areas with high contamination levels.

In the present study, length distribution of *A. crenata* showed significant regional and site-specific variations. The present study however, permitted an assessment of reasons explaining for all variations using sediment trace metals, nutrients, organic content, and particle size distribution alone, which indicate that population structure of this species likely have a complex interaction between a number of environmental factors. These including abiotic factors such as sediment particle size distribution, organic content, contaminants, food availability and abundance, nutrients, salinity, tidal level and hydrology (Bennington 1979; Marsden and Baharuddin, 2014; Marsden and Swinscoe, 2014; this study) and biotic factors like predatory pressure, inter- and intra-specific competition Therefore, it is suggested that a combination of multiple stressors is most likely responsible for determining the population structure and abundance of this species.

5.5 CONCLUSIONS

In conclusion, this study showed that *A. crenata* is highly resistant to low levels of certain trace metal contamination including Cu, Zn, Ni, Cd and Pb. Further, it showed that the sediment nutrients are playing a significant role on determining the structure of *A. crenata* populations in intertidal areas, supporting potential use of this species as an indicator of nutrient pollution. The sites were distinguished according to the population attributes (e.g. density and length) of *A. crenata* and the metals and nutrients content in sediment (Fig. 5.4). The present study however, enabled to explain all of the variations in *A. crenata* population structure, highlighted that more comprehensive studies are required to fully understand the governing factors of *A. crenata* population distribution, in turn to use population attributes of this species as indicators to monitor health conditions of estuaries in New Zealand.

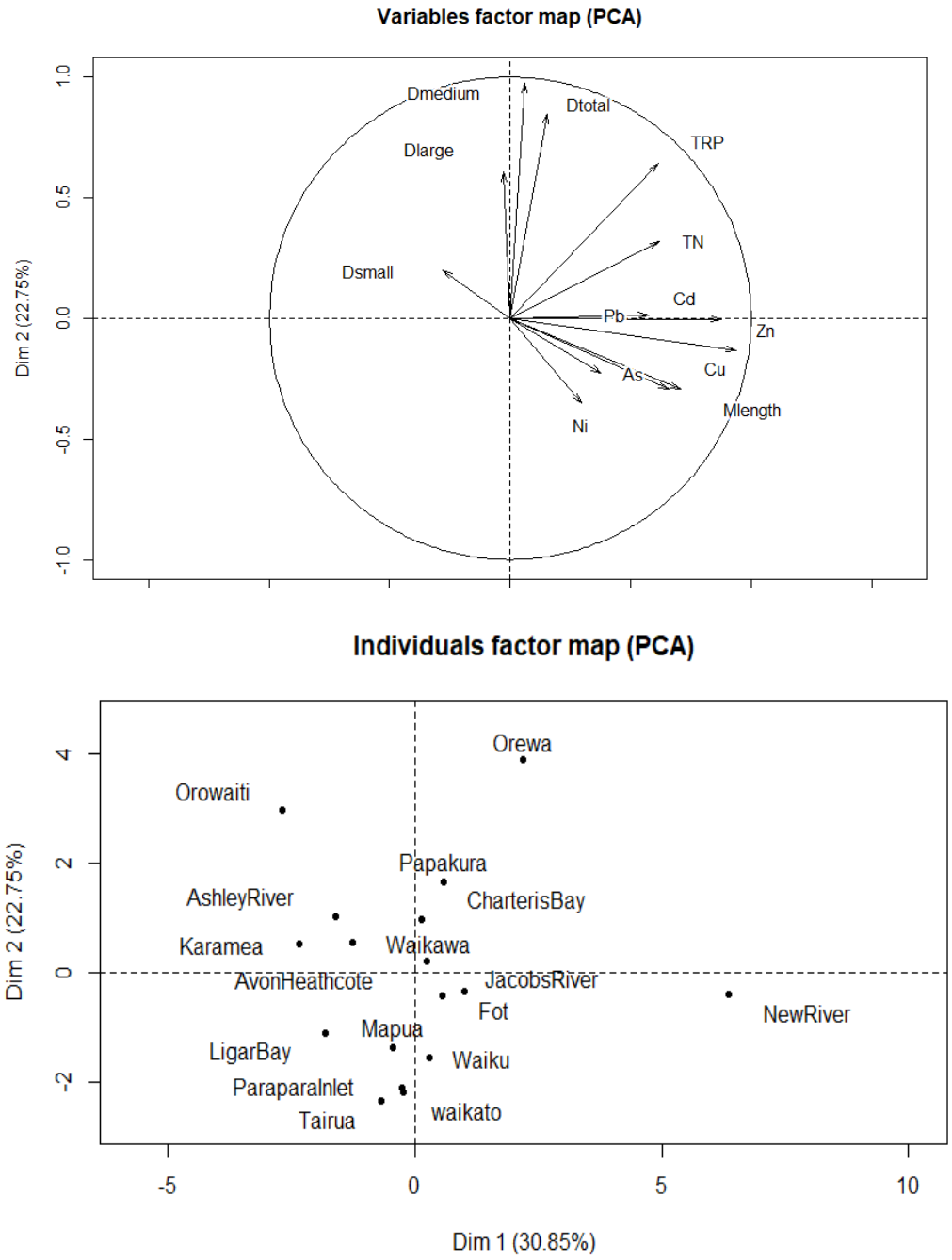


Figure 5.4 Principal component analysis (PCA) were used to distinguish sampling sites based on the population attributes (e.g. density and length) of *A. crenata* and the metals and nutrients content in sediment. Dsmall, Dmedium, Dlarge and Dtotal – density of each size class and total density, Mlength – mean length, TRP – total recoverable phosphorus, TN – total nitrogen.

Chapter 6

Assessment of *Amphibola crenata* as a bioindicator of estuarine trace metal pollution

6.1 INTRODUCTION

Estuaries are unique environments which contribute enormous ecosystem services such as maintaining water quality through natural filtration, trapping contaminants, recycling nutrients and preserving biodiversity by providing a diverse range of habitats that are critical for the survival of many aquatic species (Hallett et al., 2016). Over several decades, however, increasing anthropogenic activities have made these vital ecosystems vulnerable to various types of contaminants including trace metals, nutrients, PCB and agrochemicals (Mathews and Fisher, 2008; Marsden and Swinscoe, 2014). As a result, estuaries are now considered as amongst the most contaminated and threatened marine environments. Therefore, development of effective and efficient management techniques to assess and monitor estuarine health status is critical in order to protect and conserve those vital ecosystems.

Estuarine ecosystems are facing increasing metal pollution due to the elevated metal discharges from both point and non-point sources. Municipal effluents, industrial discharges, accidental spills, urban and agricultural runoff carry a wide range of trace metal contaminants to estuarine ecosystems worldwide (Matthiessen and Law, 2002; Mucha et al., 2003). As a result, estuarine organisms are frequently exposed to trace metal contamination and exhibit stress responses to elevated levels of contaminants. These stress responses are commonly known as "biomarkers" and can be measured and linked directly to the pollutants present.

Invertebrates are increasingly used to assess and monitor the condition of marine ecosystems (Rainbow et al., 2002: Marsden et al., 2014). For an example, mussels are used worldwide to evaluate the contaminants present in coastal habitats (e.g. Cantillo, 1998; Chandurvelan et al., 2015) and in estuaries a variety of species from different taxonomic and feeding groups have been evaluated (Marsden and Swinscoe, 2014). Deposit-feeding gastropods are particularly suitable indicators of the condition of estuarine ecosystems as they are often exposed to higher levels of contaminants than those in open coastal habitats because they are exposed not only to water borne toxins but they are also be closely associated with highly contaminated sediments.

The aim of the work discussed in this chapter was to assess the effectiveness of the deposit feeding gastropod, *Amphibola crenata* as an indicator for estuarine trace metal contamination. The hypothesis was that exposure to environmental contaminants for extended period would result in

stress adaptations which could be measured as biomarker responses. Snail samples were collected from 17 estuarine sites located on the South and North Islands of New Zealand with varying levels of anthropogenic impacts. The mudsnail was evaluated as a potential indicator species for estuarine trace metals. The study further determined which, if any, biomarkers might be suitable for detecting metal contamination in NZ estuarine ecosystems.

6.2 METHODS

6.2.1 Study area and site description

Mud snail samples were collected from six regions including Auckland, Canterbury, Nelson, Southland, Waikato, and West Coast on the South and North Islands of NZ (Figure 6.1). Each region consisted of three sampling sites except for West Coast (two sites). Sampling sites were chosen according to their different sources and degree of metal contamination. The Ashley River, Ligar Bay, Waikawa Estuary, Karamea Estuary, Taiuru Estuary, and Papakura Inlet were chosen as the reference sites for the study based on a lack of known sources of metal contamination. The sites used to collect snail samples for the present study are the same sites used for the population structure study and detailed description of the sites is provided in Chapter 5 (Table 5.1).

6.2.2 Sample collection and maintenance

Adult mud snails (n = 50; >18 mm in size) were collected at mid tide level of each sampling site. Sampling of the South Island sites was conducted during January and February 2016, while North Island sampling was conducted during February and March 2017. Snail samples were transported to the laboratory, University of Canterbury in 1.5 L polypropylene containers. Transportation was completed within 4-6 days. In the laboratory, snails were maintained in a constant temperature (15 ± 0.5°C) and photoperiod (12 hours light: 12 hours dark) in seawater that had been collected from each site. For sediment, samples from surface layer (< 2 cm in depth; n = 6) were collected into 70 ml polypropylene containers. Up reaching the laboratory, samples were stored at -20 °C until further analysis.

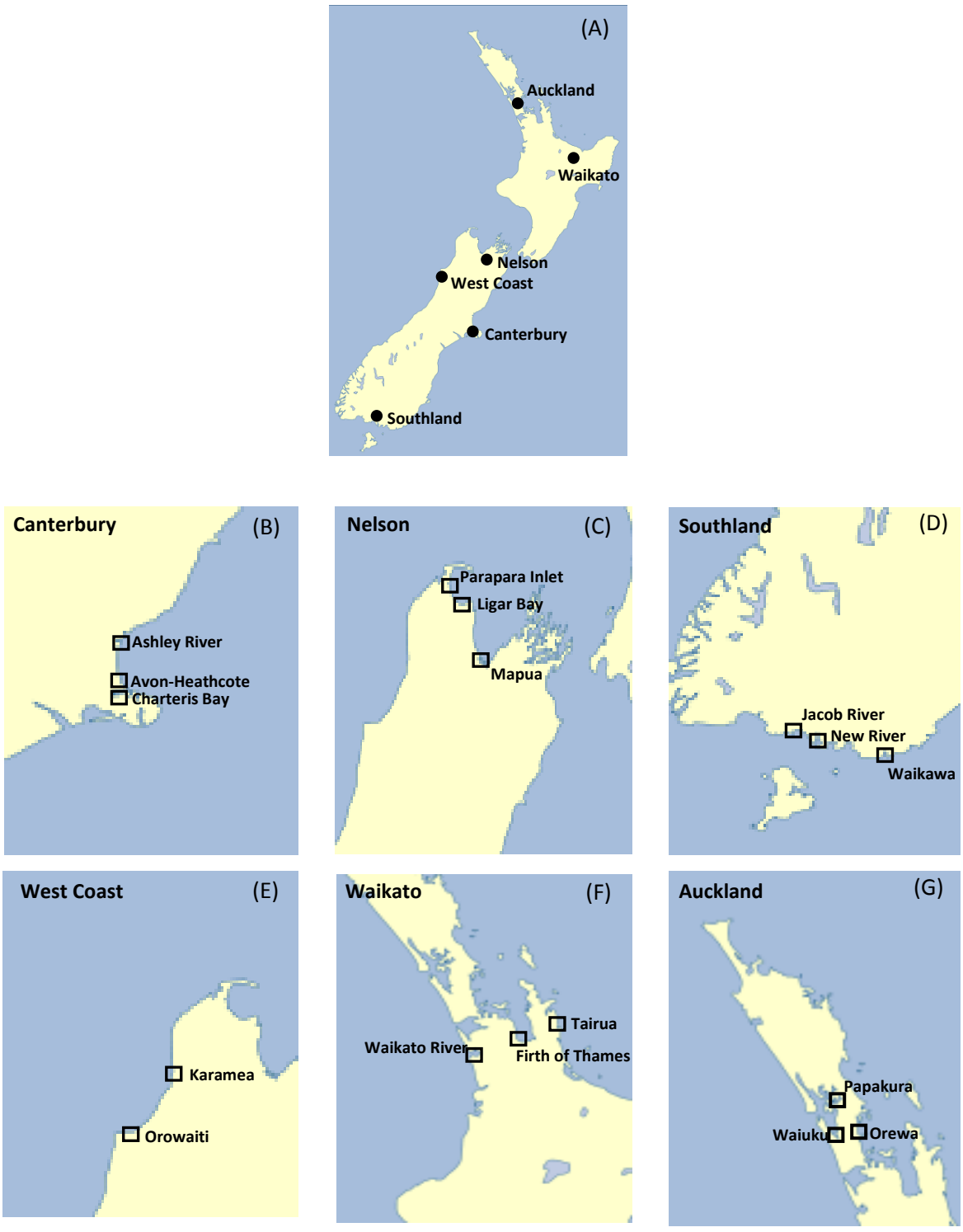


Figure 6.1 Collection sites for *A. crenata* samples for biomarker assay. (A) All regions, (B) Canterbury, (C) Nelson, (D) Southland, (E) West Coast, (F) Waikato and, (G) Auckland.

6.2.3 Tissue and sediment trace metal analysis

Snails (n = 8) from each study site were dissected and flesh was separated from the shell and stored at - 80 ºC until further analysis. After thawing, tissue samples were dried at 60 º C for 72 h and dry weights were recorded. Weighed samples were then transferred into acid-washed tubes and acid digested using 0.5 ml 70% ultrapure HNO_3 before being analysed for metals (e.g. Cu, Zn, Ni, As, Cd and Pb) content (detailed protocol available at Chapter 4, section 4.2.3). Trace metal concentrations were measured in whole body tissue and expressed as μg g^{-1} dry weight. Sediment samples collected from each site were dried at 60ºC for 72 h and were analysed for trace metals as described in Chapter 3, section 2.3.

6.2.4 Biomarker measurements

6.2.4a Physiological biomarkers

The oxygen consumption, ammonia excretion, oxygen to nitrogen ratio (O:N), and condition index (CI) were measured in individual snails (n = 6) for each sampling site. Detailed protocols for all of these measurements are provided in Chapters 2 & 4.

6.2.4b Biochemical biomarkers

The whole-body tissue of 18 snails from each site was dissected and frozen at – 80 ºC until analysis for biomarker responses. Haemolymph samples (n = 10) were collected from the foot muscle sinus and immediately analysed for haemolymph glucose and protein. After thawing, snail tissue samples from each site were analysed for catalase, lipid peroxidation, and glutathione -S-transferase activities. All biochemical biomarker measurement protocols used here were previously described in Chapters 2 & 3.

6.2.5 Statistical analyses

Different trace metal concentrations in sediment and snail tissues and the different biomarker responses between sites were analysed using a one-way nested ANOVA followed by a post-hoc test (TukeyHSD). All data were tested for, and passed, normality and homogeneity of variance

assessments using Shapiro-Wilk and Flinger-Killeen tests before being analysed. The Pearson correlation (R) was used to determine the relationship between tissue trace metals and different biomarker responses. The data which were given significant correlation coefficients were then analysed using regression analysis to determine the linear relationships between the variables. All data were processed using R statistical software (R version 3.0.2). A value of $p < 0.05$ was considered statistically significant. All data, except O:N ratio (overall mean value only), are presented as mean ± SEM, unless otherwise stated.

6.3 RESULTS

6.3.1 Trace metals in sediments

Trace metal concentrations in the sediments were site-specific resulting in significant regional and site variations (Figure 6.2). New River Estuary in the Southland recorded the highest concentrations of Cu (25 mg kg^{-1}), Zn (148 mg kg^{-1}) and Cd (0.14 mg kg^{-1}) in the sediment, whereas the highest concentrations of Ni, As, and Pb were recorded in the Mapua (59 mg kg^{-1}), Waiuku (22 mg kg^{-1}) and Firth of Thames (23 mg kg^{-1}) respectively. The Karamea and Orowaiti on the West Coast had the lowest concentrations of all trace metals tested. The level of sediment Ni in the Mapua (59 mg kg^{-1}) exceeded the Interim Sediment Quality Guidelines-High (ISQG-High; ANZECC, 2000) value of 52 mg kg^{-1}. The concentration of sediment Ni in the Parapara Inlet (28 mg kg^{-1}) and New River (29 mg kg^{-1}) exceeded the ISQG-Low value of 21 mg kg^{-1}, while the sediment As concentrations in the Waiuku (22 mg kg^{-1}) exceeded the ISQG-Low value of 20 mg kg^{-1}.

6.3.2 Trace metals in snail tissues

Accumulation of metals in *A. crenata* was site-specific resulting in significant regional differences (Figure 6.3). Analysis of tissue trace metals exhibited accumulation of high levels of As, Cu and Zn, while low levels of Cd, Ni and Pb were measured. Principally, the concentrations of trace metals in snail tissue decreased in the order Cu > Zn > As > Ni > Pb > Cd. In general, snails collected from the North Island had higher concentrations of trace metals in their tissues compared to the South Island. Analysis of correlation coefficients to test the relationships between trace

metals in the sediment and those in the snail tissue identified significant positive correlations between As ($R = 0.602$; $p < 0.05$), Cu ($R = 0.653$; $p < 0.05$), and Pb ($R = 0.546$; $p < 0.05$) concentrations in the sediment and the tissue (Table 6.1).

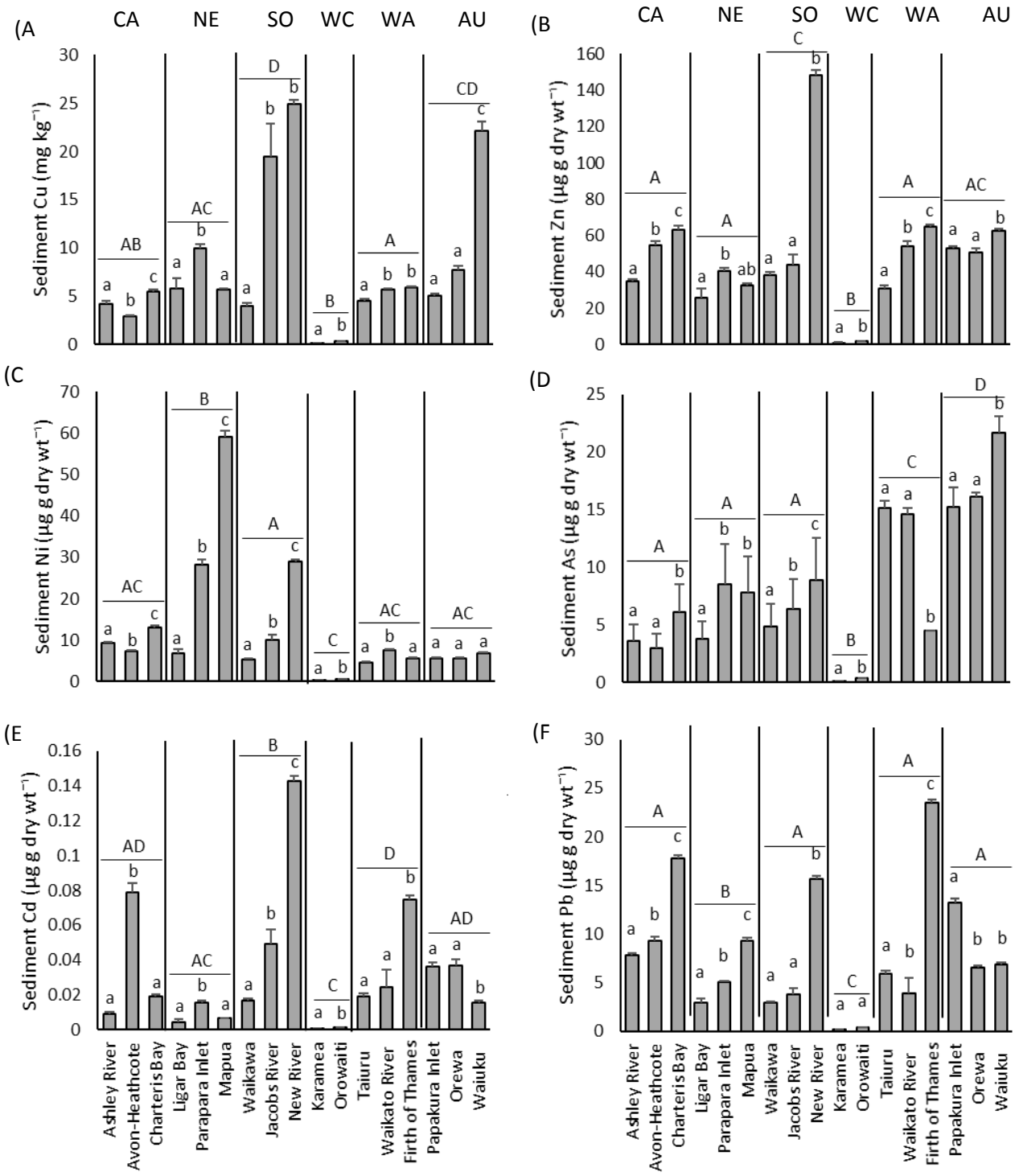


Figure 6.2 Metal concentrations measured in the sediment from the seventeen sampling sites within four regions in the South Island and two regions in the North Island of NZ. Cu (A); Zn (B); Ni (C); As (D); Cd (E) and, Pb (F). Plotted values represent mean ± SEM of 6 replicates. CA – Canterbury, NE – Nelson, SO – Southland, WC – West Coast, WA – Waikato and AU – Auckland. Upper case letters indicate significant differences between regions, while lower case letters displayed significant differences between sites within each region as determine by one-way nested ANOVA followed by Tukey post-hoc test at $p < 0.05$.

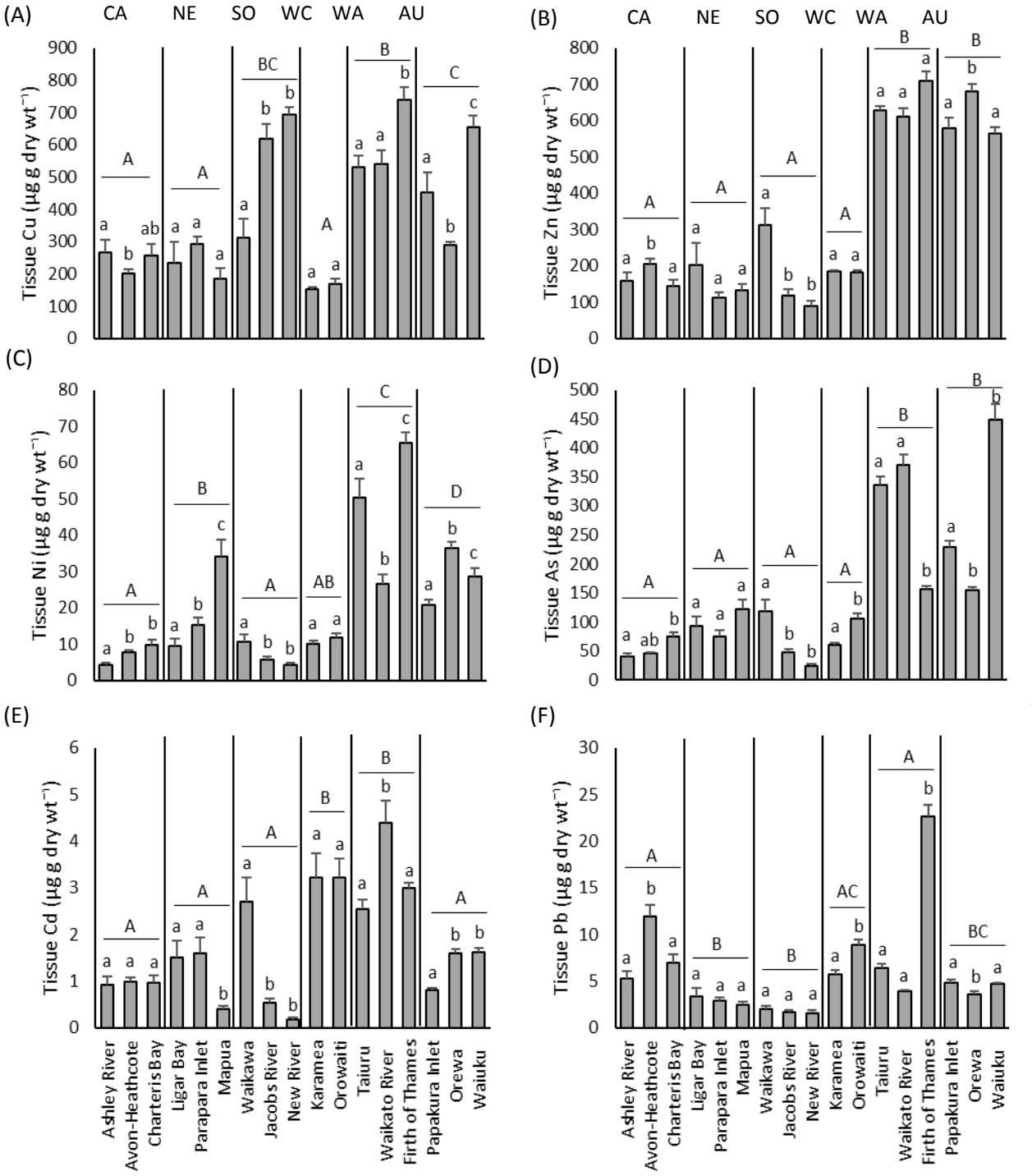


Figure 6.3 Metal concentrations measured in the tissues of *A. crenata* from the 17 sampling sites within four regions in the South Island and two regions in the North Island of NZ. Cu (A); Zn (B); Ni (C); As (D); Cd (E) and, Pb (F). Plotted values represent mean ± SEM of 6 replicates. Upper case letters indicate significant differences between regions, while lower case letters displayed significant differences between sites within each region as determine by one-way nested ANOVA followed by Tukey post-hoc test at $p < 0.05$.

6.3.3 Physiological responses

Oxygen consumption of snails, with the exception of the Waikato Region, showed no significant regional or site-specific variations (Figure 6.4A). There was, however, a significant positive correlation with sediment Pb concentration ($R^2 = 0.3$; $p < 0.05$; Table 6.2). The excretion rate of *A. crenata* displayed significant site-specific and regional differences with a significantly higher excretion rate for most of the polluted sites compared to the corresponding reference sites (Figure 6.4B).

Snails from the Auckland and Waikato Regions (North Island) had higher excretion rates than those from all other sites in the South Island. Correlation coefficients calculated to test the relationships between trace metals in the snail tissue versus excretion rate showed significant positive correlations between sediment arsenic ($R^2 = 0.31$; $p < 0.05$; Table 6.2) concentration and the excretion rate. The O:N ratio based on the oxygen consumption and excretion rate were low for most of the polluted sites compared to the corresponding reference sites (Figure 6.5A). For the CI there were few significant differences amongst sites and regions (Figure 6.5B). The CI of snails were mostly lower in the Southland, Waikato and Auckland Regions, CI highest on the West Coast. The CI was negatively correlated to As ($R^2 = 0.40$; $p < 0.01$), Cd ($R^2 = 0.36$; $p < 0.05$), Cu ($R^2 = 0.31$; $p < 0.05$) and Zn ($R^2 = 0.48$; $p < 0.01$) concentration in the tissue.

Table 6.1 Relationship of trace metal concentration between sediment and mud snail soft tissues.

Metal	Coefficient of determination (R^2)	Equation for the line of best fit	p- value
Cu	0.43	y= 20.5x + 237.5	**< 0.01**
Zn	0.001	y= -0.25x + 341.4	0.89
Ni	-0.06	y= 0.01x + 20.5	0.97
As	0.36	y= 14.7x + 36.1	**< 0.05**
Cd	0.11	y= -11.2x + 2.1	0.18
Pb	0.3	y= 0.4x + 2.3	**< 0.05**

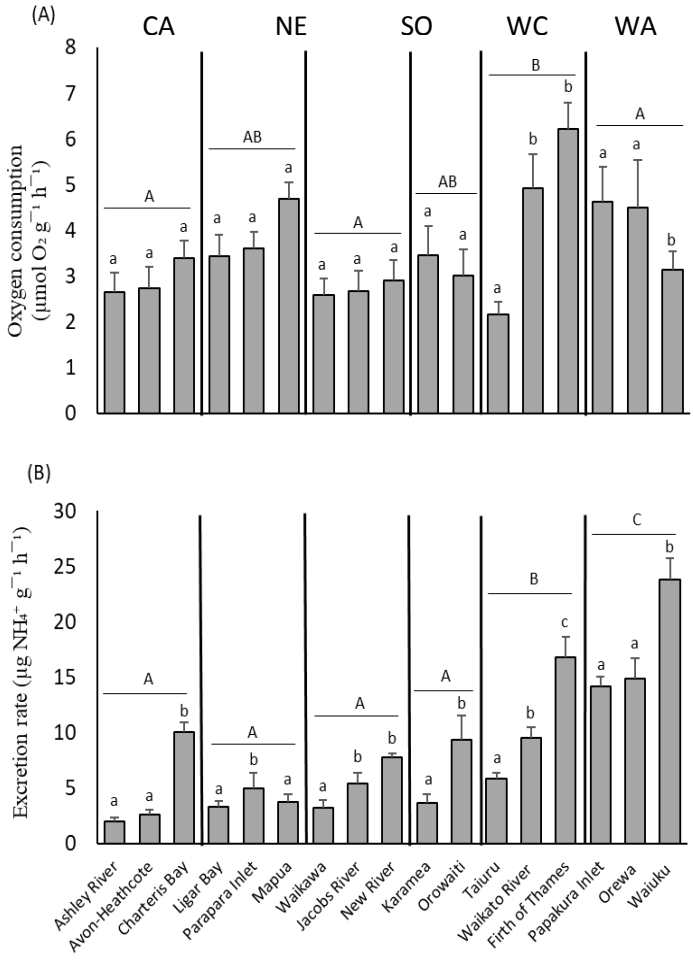


Figure 6.4 Oxygen consumption (A) and excretion rate (B) of *A. crenata* from different sites. Plotted values represent mean ± SEM of 6-5 replicates. Upper case letters indicate significant differences between regions, while lower case letters displayed significant differences between sites within each region as determine by one-way nested ANOVA followed by Tukey post-hoc test at $p < 0.05$.

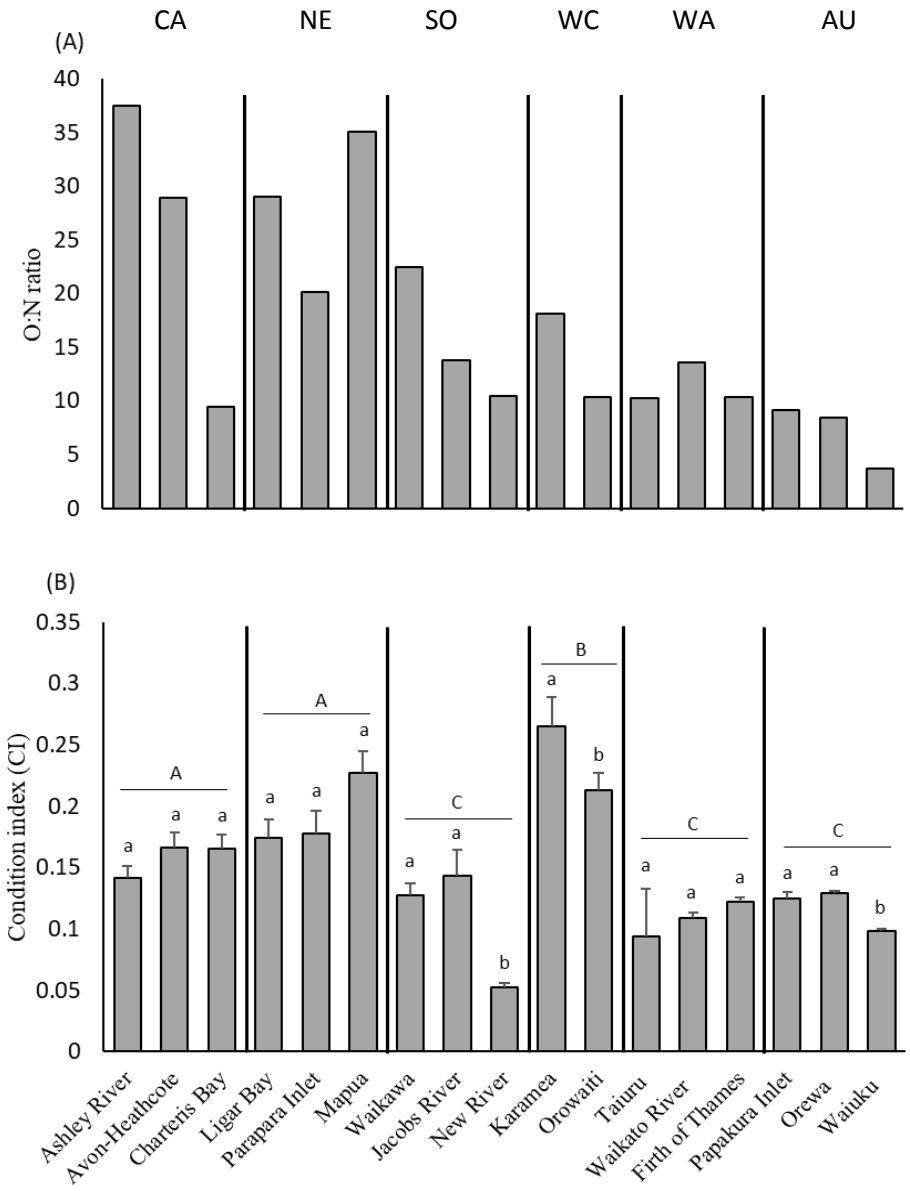


Figure 6.5 O:N ratio (A) and condition index (CI) (B) of *A. crenata* from different sites. Plotted values represent mean ± SEM of 6-5 replicates. Upper case letters in the lower panel indicate significant differences between regions, while lower case letters indicate significant differences between sites within each region as determine by one-way nested ANOVA followed by Tukey post-hoc test. at $p < 0.05$.

6.3.4 Biochemical responses

With the exception of Orowaiti in the West Coast and the Waikato River in the Waikato Region, snails from other polluted sites had significantly high levels of tissue catalase than snails from the corresponding reference sites (Figure 3.6A). There were no significant regional differences with respect to catalase activity. Conversely, elevated levels of lipid peroxidation were observed in the Waikato and Auckland Regions in the North Island compared to the regions in the South Island. Within the Waikato and Auckland Regions, all polluted sites exhibited significantly high levels of lipid peroxidation than reference sites (Figure 3.6B). Tissue catalase activity was correlated with Cd ($R^2 = 0.32$; $p < 0.05$; Table 6.2) concentration in the snail tissue. Lipid peroxidation increased with tissue Cd ($R^2 = 0.35$; $p < 0.05$) and Pb ($R^2 = 0.25$; $p < 0.05$; Table 6.2) concentrations in the snail tissue. GST activity of snails showed significant regional differences as higher tissue GST levels were observed in snails collected from the West Coast, Southland, Auckland and Waikato Regions, while lower values were recorded for snails from the Canterbury and Nelson regions (Figure 6.6C). Tissue GST levels showed a significant positive correlation to Cd ($R^2 = 0.23$; $p < 0.05$; Table 6.2) concentration in the snail tissue.

Haemolymph glucose was significantly higher in the Auckland, Charteris Bay, Southland and Waikato snails than in snails collected from the Canterbury and West Coast Regions, while the West Coast snails reported the lowest haemolymph glucose concentrations (Figure 6.7A). The New River site snail haemolymph glucose concentrations were the highest (341 ± 29 mg mL^{-1}) reported for any of the 17 sites flowed by snails from Waiuku (322 ± 56 mg mL^{-1}). Haemolymph glucose concentrations displayed significant positive correlations to As ($R^2 = 0.39$; $p < 0.05$), Cu ($R^2 = 0.49$; $p < 0.01$), Ni ($R^2 = 0.23$; $p < 0.05$) and Zn ($R^2 = 0.27$; $p < 0.01$) concentrations in the snail tissue.

In contrast with the above results, haemolymph protein was significantly lower in the Auckland, Waikato and West Coast snails than those collected from the Canterbury, Nelson and Southland Regions (Figure 6.7B). Nevertheless, except for the New River, there were no significant site-specific differences with respect to haemolymph protein concentration, while haemolymph protein was negatively correlated to As ($R^2 = 0.22$; $p < 0.05$), Cd ($R = 0.53$; $p < 0.01$) and Zn ($R^2 = 0.33$; $p < 0.05$) concentrations in the snail tissue (Table 6.2).

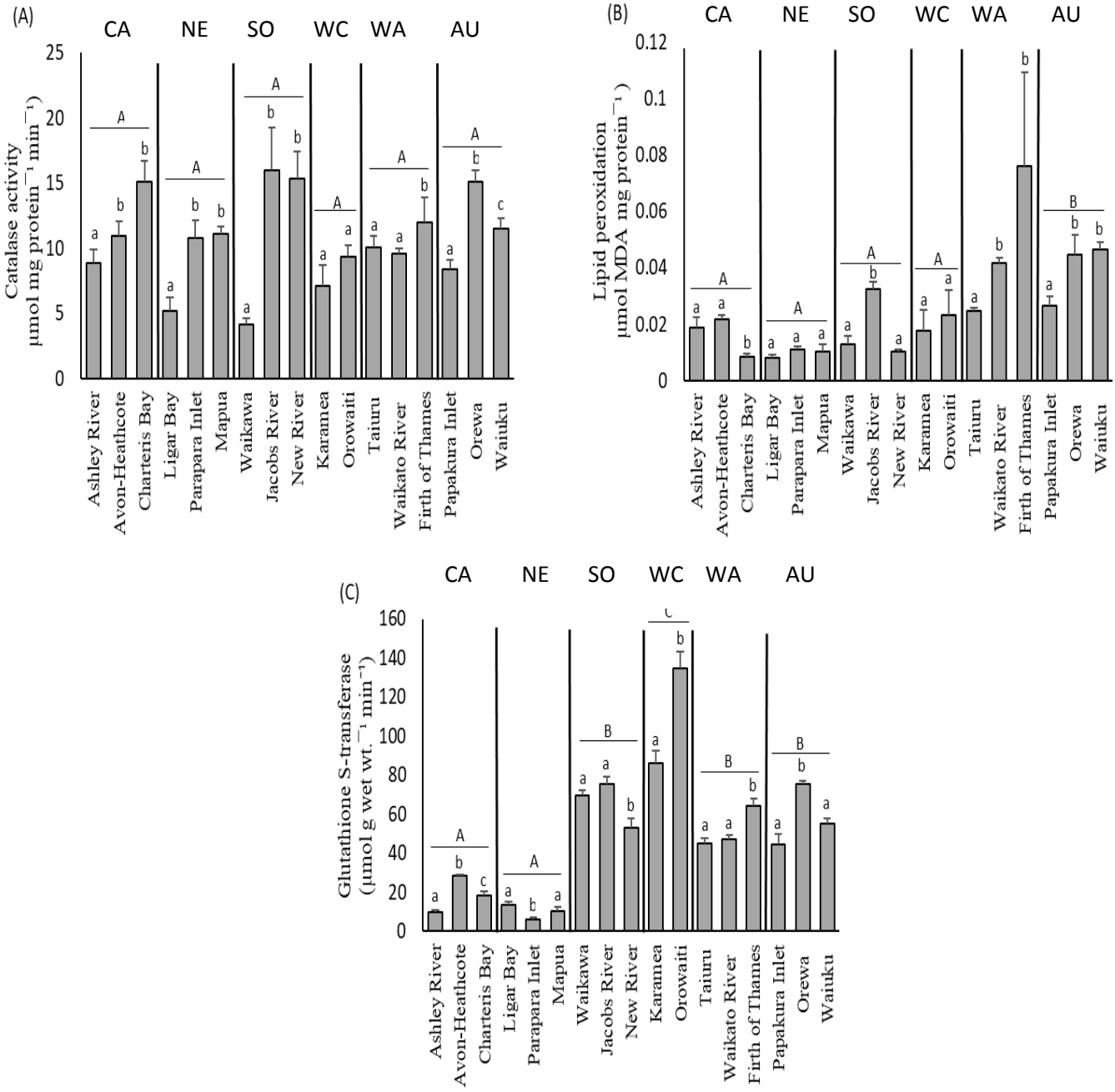


Figure 6.6 Responses of catalase activity (A); lipid peroxidation (B) and glutathione -S-transferase (C) of *A. crenata* from different sites. Plotted values represent mean ± SEM of 6-5 replicates. Upper case letters in the lower panel indicate significant differences between regions, while lower case letters displayed significant differences between sites within each region as determine by one-way nested ANOVA followed by Tukey post-hoc test. at $p < 0.05$.

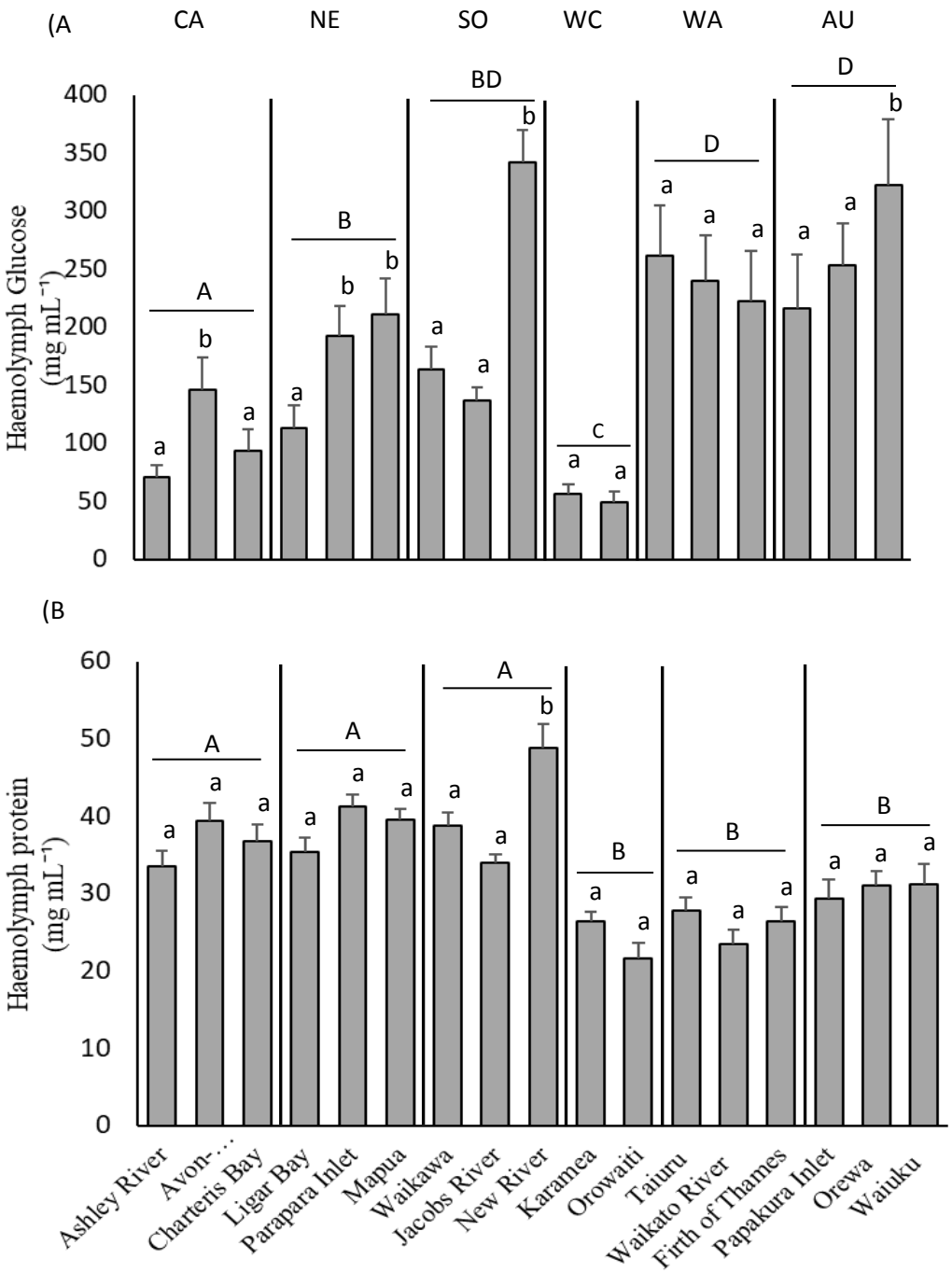


Figure 6.7 Biochemical responses of *A. crenata* collected from different sampling sites. Haemolymph glucose (A) and haemolymph protein (B). Plotted values represent mean ± SEM of 6-5 replicates. Upper case letters in the lower panel indicate significant differences between regions, while lower case letters displayed significant differences between sites within each region as determine by one-way nested ANOVA followed by Tukey post-hoc test. at $p < 0.05$.

Table 6.2 Relationship between physiological and biochemical responses, and tissue trace metal concentrations (whole body tissue) or metal exposure concentration (physiological measures).

Tissue metal	Biomarker	Coefficient of determination (R^2)	Equation for the line of best fit	p- value
Cu	Condition index	0.31	y= -0.005x + 0.2	< 0.05
	Haemolymph glucose	0.49	y= 0.3x + 61.6	< 0.01
Zn	Condition index	0.48	y= -0.001x + 0.2	< 0.01
	Haemolymph glucose	0.27	y= 0.19x + 117.3	< 0.05
	Haemolymph protein	0.33	y= -0.02x + 38.9	< 0.05
Ni	Haemolymph glucose	0.23	y= 2.4x + 131.6	< 0.05
As	Excretion rate	0.31	y= 0.03x + 4.7	< 0.05
	O:N ration	0.21	y=-0.91x + 23.9	< 0.05
	Condition index	0.40	y= -0.006x + 0.2	< 0.01
	Haemolymph glucose	0.39	y= 0.39x + 123.8	< 0.05
	Haemolymph protein	0.23	y -0.0003x + 1.6	< 0.05
Cd	Catalase activity	0.32	y= -1.3x + 12.9	< 0.05
	Condition index	0.36	y= -0.86x + 0.2	< 0.05
	Lipid peroxidation	0.35	y= 0.01x + 0.008	< 0.05
	glutathione S-transfereas	0.23	y= 13.5x + 25.2	< 0.05
	Haemolymph protein	0.53	y= -4.3x + 40.9	< 0.01
Pb	Oxygen consumption	0.3	y = 0.09x + 2.9	< 0.05
	Lipid peroxidation	0.25	y= 0.002x + 0.02	< 0.05

6.4 DISCUSSION

The current study showed that the trace metal concentrations in the sediments and mud snail tissues varied widely between regions as well as sites within a region. In general, snails collected from sites with elevated sediment trace metal concentrations had high concentrations of metals in their tissues and exhibited wide range of variations in physiological and biochemical responses. Therefore, it is suggesting that metal accumulation in tissues, physiological and biochemical responses of *A. crenata* could be potentially use as an indicators of environment contamination.

6.4.1 Trace metal concentration in sediments

Surface sediments are recognised as indicators of environmental conditions and sediment trace metal content is regularly used to identify anthropogenic inputs into aquatic systems (Rodriguez-Barroso et al., 2010; Chandurvelan et al., 2015). For most of the sites, the spatial distribution of sediment trace metals found in this study were generally similar to those found in previous studies (Bolton-Ritchie, 2008; Cavanagh and Ward, 2014; Marsden and Baharuddin, 2014, Chandurvelan et al., 2015). Of the metals analysed in the sediments, Zn (0.9 – 148.3 mg kg^{-1}) was found to be the most abundant metal. The exceptions were for Ni concentrations in Mapua and Parapara Inlet in Nelson Region, New River in Southland Region and As concentration in Waiuku in Auckland Region. All other sites had concentrations of sediment trace metals that were below the ISQG-Low trigger values. In general, trace metal concentration in sites in the North Island sediment (i.e. Auckland and Waikato) followed the order: Zn > As > Ni > Cu > Pb > Cd, whereas in South Island sediment (i.e. Canterbury, Nelson, Southland and West Coast) were found to be in the following descending order: Zn > Ni > Cu > Pb > As > Cd. The high concentration of As in Auckland and Waikato region estuarine sites could be associated with input from geological sources rather than anthropogenic impacts (Park, 2014; Mills, 2014). Sediment trace metals for West Coast sites (Karamea and Orowaiti) had the lowest values, consistent with less human anthropogenic activities.

6.4.2 Trace metal concentration in *A. crenata* tissues

Gastropods are recognised as net accumulators of trace metals from their surroundings and this was found in the present study for the *A. crenata*. In general, the concentrations of trace metals in *A. crenata* tissues were higher than those found in previous studies of field-exposed gastropods (Nicolaodou and Nott, 1998; Conti et al., 2006; Maher et al., 2016). In the present study, a general pattern of metal accumulation in snail tissues were found to be in the following descending order: Cu > Zn > As > Ni > Pb > Cd and were consistent with other gastropods (Yap and Cheng, 2013; Kupekar and Kulkarni, 2014). The lower accumulation levels of highly toxic elements including Ni, Pb and Cd suggest the presence of a variety of homeostatic mechanisms such as excretory pathways that may limit their accumulation. In contrast, As, which is considered to be a toxic element, had relatively high accumulation levels in *A. crenata* tissues. Similarly, high levels of As were found in green lipped mussel, *Perna canaliculus* and those authors concluded this could be attributed to regulatory function of As in mussel osmoregulation (Whaley-Martin et al., 2012: Chandurvelan et al., 2015).

In the present study, the concentrations of As, Cu, and Pb in the sediment and the whole-body tissue of *A. crenata* were correlated ($R^2 = 0.36$, $R^2 = 0.43$ and $R^2 = 0.3$ respectively). The lack of a correlation between other trace metals (e.g. Cd, Ni and Zn) in the sediment and in the tissues suggests that metal uptake of *A. crenata* may occur through other alternative pathways (e.g. via food or seawater), or may be regulated.

6.4.3 Physical biomarkers

Trace metals in aquatic environments can have adverse affect on oxygen consumption in aquatic organisms (Chinni et al., 2000; Vijayavel et al., 2007; Chandurvelan et al., 2012). Laboratory-based, short-term and long-term studies have shown that oxygen consumption of *A. crenata* was negatively correlated with tissue burdens of Cd, and thus it has been proposed that this physiological biomarker may be of utility as a bioindicator of metal exposure (Chapters 2 & 3). In contrast to the laboratory findings, snails collected from the Firth of Thames in the Waikato Region had the highest oxygen consumption rate, where the highest level of tissue Cu, Ni, Pb, and Zn

levels were found. This would suggest that under environmentally relevant, but elevated, levels of contaminants *A. crenata* may have the ability to increase their metabolic processes resulting on increased oxygen consumption rate. Similar results were observed for the estuarine fiddler crab *Uca rapax* exposed to trace metal contaminants (Capparelli et al., 2016).

Ammonium is the final product following catabolism of protein and amino acids. In the present study, snails collected from the majority of the polluted sites had high levels of ammonia excretion, reflecting an increase in catabolism of protein and amino acids. These findings are consistent with previous laboratory studies of mollusc groups (e.g. Wu and Chen, 2004, Chandurvelan et al., 2012). It is probable that an elevation in ammonia excretion was related to either enhanced degradation of protein damaged by trace metal exposure, or a change in energy metabolism as a result of metal exposure (Chapter 2). Support for the hypothesis that there is a switch to protein metabolism in snails exposed to elevated levels of trace metals, is provided by the observed low values of O:N ratios for snails collected from the polluted sites in the present study. Low O:N ratios indicate protein-dominated catabolism (Mayzaud and Conover, 1988).

The condition index (CI) is regarded as one of the best indicators of gross body state for environmental studies (Hyotylanen et al., 2002), and is widely used to assess the health of aquatic organisms exposed to environmental contamination (Bodin et al., 2004; Norkko and Thrush, 2006; Marsden et al., 2014). In the present study, low CI values were observed for the snails collected from polluted sites in the Auckland, Southland, and Waikato Regions, where high metal bioaccumulation occurred. These results suggest that the mud snails with the highest accumulated metal loads have paid a physiological cost in the storage detoxification process underlying their metal accumulation. Previous research on *A. crenata* has also confirmed that this species exhibited changes in CI due to environmental contamination (Marsden and Baharuddin, 2014). The CI of *A. crenata* was negatively correlated with most of the sediment trace metals (e.g. As, Cd, Cu, and Zn; Table 6.2), reflecting potential utility in CI of *A. crenata* as a biomarker of trace metal pollution.

6.4.4 Biochemical biomarkers

Catalase is an important and sensitive biomarker of oxidative stress and is the primary defense against oxidative damage (Aboul-Ela et al., 2011). In the present study, induced catalase activity

in snails in polluted sites may reflect the cumulative effects of multiple stressors including both trace metals and other pollutants. Increased concentrations of catalase have been observed in wide range of aquatic organisms exposed in polluted areas when compared to less polluted areas (Vlahogianni et al., 2007; Siwela et al., 2010; El-Shenawy et al., 2012). The observed high lipid peroxidation levels in snails from the Waikato and Auckland Regions suggest that the induced catalase increases in snails were insufficient to reduce lipid peroxidation levels in those polluted areas. This provides further evidence that the North Island estuarine sites are more contaminated than South Island sites. Similar results were reported for the mussel, *Mytilus galloprovincialis* collected from metal contaminated coastal areas in Greece (Vlahogianni et al., 2007). Chandurvelan et al., (2015) also reported elevated lipid peroxidation levels in the mussel, *Perna canaliculus* collected from metal contaminated coastal areas in New Zealand. Overall, variations in tissue catalase and lipid peroxidation levels in *A. crenata* in relation to the trace metal contamination suggests that these chemicals could be utilized as biomarkers to detect trace metal pollution in NZ estuaries using *A. crenata*.

The GST are a group of detoxifying enzymes whose main function is to convert endogenous and xenobiotic compounds to water soluble intermediates that may be eliminated (Dailianis, 2010). In the present study, elevated levels of GST activity were reported for *A. crenata* from contaminated areas and this is consistent with previous studies for other gastropods. A number of studies have shown elevated activity of GST in aquatic gastropods when exposed to trace metal contamination. For example, El-Shenawy et al., (2012) reported elevated levels of GST activity in the pulmonate gastropod, *Eobania vermiculata* collected from polluted areas. Abdel-Halim et a., (2013) also found increased level of GST activity in the terrestrial gastropod, *Helix aspersa* collected from sites contaminated with trace metals. Klimova et al. (2017) found elevated levels of GST activity in the zebra mussel, *Dreissena polymorpha*.

In recent years, haemolymph metabolites such as protein and glucose have been identified as indicators of physiological condition in organisms exposed to different environmental conditions (Rosas et al., 2004; 2007). Haemolymph glucose concentrations in aquatic organisms have been recognised as a potential biomarker for a variety of anthropogenic stressors including trace metals, anoxia, and elevated CO_2 (Hall and van Ham, 1998; Lorenzon, 2005; Hannan et al., 2016).

Glucose in animal haemolymph can be derived from stored glycogen (Cameselle et al., 1980) or exogenous sources such as the diet (Silva and Wright, 1992). The results from the present study showed that haemolymph glucose levels rose in response to elevated concentrations of trace metals in the environment, suggesting hyperglycemic responses in *A. crenata*. This hyperglycemia may be associated with biosynthesis of glucose from precursors other than lipids/carbohydrates, especially from protein (gluconeogenesis) (Bislimi et al., 2013). This result further supports the hypothesis that there is a switch to protein metabolism in metal-exposed mud snails. In contrast, Bislimi et al. (2013) found reduced levels of haemolymph glucose (hypoglycemia) in the garden snail, *Helix pomatia* exposed to industrial pollution. The effects of anthropogenic stressors on haemolymph glucose may, therefore, vary greatly depending on the species and type of the stressor.

Haemolymph proteins play a vital role in the animal physiology such as transporting O_2, and maintaining osmotic pressure (Lorenzon et al., 2011). In the present study, haemolymph protein levels of snails showed significant regional differences as snails from the North Island sites displayed low levels of haemolymph protein than those from the South Island sites. These findings reflect that snails in the North Island sites may experience higher environmental stress than those in the South Island sites. Rajan et al., (2012) reported a decline level of haemolymph protein in the fresh water crab, *Paratelphusa jacquemontii* exposed to environmentally relevant Cu concentrations. In another study, Bislimi et al. (2013) also found declining haemolymph protein in the garden snail. *Helix aspersa*, collected from sites contaminated with trace metals of Cd, Fe, Pb and Zn. In contrast, Rosas et al. (2004) reported elevated levels of haemolymph protein in the shrimp, *Litopenaeus setiferus* exposed to multiple environmental stressors.

In addition, both haemolymph glucose and protein displayed a significant correlation with most of the trace metals in snail tissues (for glucose; Cu, Zn, Ni and As and for protein; Zn, As and Cd; Table 6.2) suggesting that these haemolymph metabolites in mud snails can be used as potential biomarkers of toxicity for long-term monitoring in estuarine ecosystems in New Zealand. A number of studies have reported a positive correlation between haemolymph glucose in aquatic organisms and trace metals in the environment. For example, Lorenzon et al., (2000) reported significant positive correlation in haemolymph glucose concentration and waterborne Cd, Cu and Hg in shrimp, *Palaemon elegans*, while Jiang et al. (2013) found a positive correlation between

haemolymph glucose and waterborne Cd in freshwater crayfish, *Cherax quadricarinatus*. But no similar studies have been reported for haemolymph protein.

6.5 CONCLUSIONS

The present study was the first evaluation of an estuarine gastropod species as a potential bioindicator of contaminants in which multiple biomarkers were used. Overall, the sites were distinguished according to the metal content of the snail tissues, metal contamination in sediment, and biomarker responses. The trace metal accumulation in body tissues varied with concentration of those metals in the environment, indicating that the mud snail is a suitable bioindicator for estuarine metal contamination. Of the biomarkers examined in this study, those with the greatest promise for detecting environmental impact related to metal contamination included the condition index, catalase activity, lipid peroxidation and haemolymph glucose and protein. This study did not, however, measure other contaminants present in the estuarine systems which could have had a significant influence on the snail physiological and biochemical responses.

Chapter 7

General Discussion

The research in this thesis had the following aims, (a) determine toxic mechanisms of *A. crenata* to both acute and chronic Cd exposure, and to investigate Cd bioaccumulation in different tissues as a function of exposure levels and time (b) assess multiple biomarker responses of field-collected snails in relation to the trace metal and nutrients pollution and, (c) evaluate the potential utility of *A. crenata* as a bioindicator for estuarine contamination in NZ. This discussion is directed towards the role of *A. crenata* as a monitoring tool, some methodological considerations, and future research directions.

7.1 The mud snail, *Amphibola crenata* as a monitoring tool for coastal contamination

The toxicological, physiological, and biochemical effect of Cd in mud snail, *A. crenata* at different concentrations and duration of exposure were investigated by conducting laboratory based acute (48 h) and chronic (21 days) toxicity tests. This thesis has shown that *A. crenata* is relatively tolerant to waterborne Cd toxicity. This tolerance to Cd could be explained by the presence of large internal energy reservoirs (e.g. glycogen reserves), efficient antioxidant defences (e.g. catalase activity) and mechanisms that possibly restrict Cd exposure (e.g. mucus production and/or opercular closure). Exposure to Cd induced a cascade of physiological and biochemical responses in mud snails. Some of such biomarker responses include induced excretion rate, catalase activity, haemolymph glucose and protein and declined oxygen consumption, O:N ratio and glycogen reserves. Overall the laboratory-based Cd toxicity studies have clearly indicated that the effects of Cd on biomarker responses were tissue-specific, dose- and time-dependent.

Metal concentrations in snail tissues (e.g. As, Cd, Cu, Ni, Pb, and Zn) were measured to assess the utility of mud snails as bioindicator for coastal metal pollution in NZ. The sites were distinguished according to the metal content of the snail tissues, metal contamination in sediment, and biomarker responses. Of the biomarkers examined in field-collected mud snails, those with the greatest promise for detecting environmental impact, related to metal contamination, included the condition index, glutathione-S-transferase, catalase activity, lipid peroxidation, haemolymph glucose and protein. The study on population attributes (e.g. density and length) of *A. crenata* has indicated significant site-specific and regional differences in snail population structure and significant positive correlations between sediment nutrients, particularly total recoverable phosphorous, to

snail density and shell length characteristics (e.g. minimum, mean, median and maximum length) and sediment trace metals. Overall, the field studies indicated that the *A. crenata* has the potential to be used as a bioindicator for assessment of coastal contamination in NZ.

Bivalve molluscs including mussels, oysters and clams have been widely analysed for toxic metal levels. This has been to ensure shellfish food safety and as trace metal biomonitors to assess the environmental health of coastal ecosystems (Marsden et al, 2014). Detritus and deposit feeding amphipods have been successfully used as metal bioindicator of contaminants in estuarine habitats in Europe and New Zealand (Marsden et al., 2003; Marsden et al., 2004; Peake et al., 2006). Mussels are effective biomonitors of trace metal contaminants in estuarine and coastal and rocky shores (Chandurvelan et al., 2012, 2013). There have been no comprehensive biomarker studies on animals which live in or on intertidal mudflats. This thesis research was the first detailed biomarker study on an intertidal gastropod species in New Zealand.

Estuaries are the most contaminated ecosystems in New Zealand when compared to the coastal and freshwater ecosystems because they are the major dumping sites of urban, agricultural and industrial wastes. As a result, published reports have shown a continuous decline of environmental health conditions of New Zealand estuaries. Currently, many of the estuarine health monitoring programmes in New Zealand involve measuring the concentrations of major chemicals in the water and sediments. This approach does not account for the ongoing anthropogenic effects to the estuarine biota. In addition, there are limitations with chemical monitoring that reduces the usefulness for environmental management.

Some Regional Councils in New Zealand have used cockles as a model species to monitor estuarine health conditions. This species, however, shows highly patchy distribution within estuaries which limits their usefulness as a biomonitor (Cavanagh and Ward, 2014). Therefore, the present thesis highlighted the importance of mud snail, *A. crenata* as a model species for estuarine monitoring in NZ considering various aspects.

Firstly, mud snails fulfill most of the criteria required as a bioindicator: 1) they are widely distributed throughout NZ estuaries and occur in both contaminated and non-contaminated areas, allowing the evaluation of site-specific and regional variations on the effects of contaminants on estuarine biota, 2) they are present in high abundances throughout the year, allowing comparison

of seasonal and temporal variations in contaminant inputs, 3) they have a long-life cycle (nearly 10 years; Shumway, 1981) which allows investigation of a historical record of contaminants throughout the organism life history. Second, in the present thesis both the laboratory and field experiments have shown that toxicological, biochemical, physiological and population attributes of this species were significantly correlated with environmental contaminants. For example, *A. crenata* is a net accumulator of trace metals as reflected in the observed relationships between sediment and tissue trace metals and metal pollution index (MPI) of the sediment and tissues. Of the metal and nutrient biomarkers examined in this study, those with the greatest promise for detecting environmental impact included population attributes (e.g. density and length), condition index, catalase activity, lipid peroxidation and haemolymph glucose and protein. Of these population attributes of *A. crenata* can be easily measured in the field. It is a simple and cost-effective tool which environmental managers can use to make decisions on site selections (e.g. to distinguish between contaminated non-contaminated and eutrophicated sites within an estuary or between estuaries) for future monitoring.

The findings from the laboratory studies demonstrated a strong biomarker response in the viscera (i.e. gonad and digestive gland) of mud snails. For example, metal levels and catalase activity in viscera were higher than the foot muscle and remaining tissues, whereas glycogen level was lower, suggesting digestive gland is the major organ involved in storage and detoxification of metals in *A. crenata*. Therefore, this thesis highlighted the potential utility of digestive gland (or viscera) for biomarker measurements in *A. crenata* for future monitoring studies.

This thesis also provided information on toxic mechanisms of *A. crenata* under both acute and chronic Cd exposures. From an ecotoxicological perspective, understanding of toxic mechanisms is essential for environmental risk assessment. Mechanistic data are also increasingly important for the development of regulatory tools (e.g. Biotic Ligand Model; Di Toro et al., 2010), which may help environmental decision makers to rapidly assess risk associated with toxic metals.

Overall, the main implication of the present thesis research is the possible use of *A. crenata* as a biomonitoring tool for New Zealand estuaries. This uses the biological effects, caused by anthropogenic contaminants in estuarine ecosystems, and will provide useful information for environmental managers.

7.2 Methodological considerations

In the acute Cd experiment, Cd exposure concentrations were significantly in excess of those likely to be found in natural settings and could potentially overwhelm the capacity of organism to regulate metal accumulation. These types of studies however, facilitate site-specific determination of toxicity based on knowledge of water chemistry and mechanisms of uptake. They also provide an overview of the mechanisms of toxicity and/or defense. Cd concentrations used in the chronic exposure (21 days) test were likely to be more representative of the environmental exposure scenarios. The longer exposure period under chronic experimental settings, permits us to elucidate mechanisms that allow the organism to adapt stressful environmental conditions. In the present study, the toxicity of Cd to *A. crenata* was studied using an aqueous solution rather than using experimentally dosed sediments. Laboratory studies on the uptake of trace metals from sediment are difficult to interpret due to issues with determining the extent to which metal ions become bonded to the sediment and the proportion of uptake through ingestion of organically-bound metals (Bennington, 1979).

In the present study, all of the mud snails used for the laboratory studies were acclimated before being used for the experiments and they were not fed. This was in order to preclude any unwanted variations in their physiology prior to the experiments. Consequently, there is a possibility that starvation may have had an effect on mud snail physiology and metabolic activities. In addition, in any laboratory experiment, organisms are being removed from their natural environments. It is difficult to know whether those organisms are in any way stressed and therefore whether they are behaving or functioning normally. In the field-base experiments, this study only measured availability of trace metals and nutrients in the environment and did not measure other contaminants present in the estuarine systems.

7.3 Future research recommendations

The present study investigated cadmium compartmentalization in different tissues in *A. crenata* in relation to both the exposure concentration and duration of exposure. The study further investigated accumulation of different trace metals (e.g. Cu, Zn, Ni, As, Cd and Pb) in *A. crenata* soft tissues. Some studies on gastropod shell materials have also been conducted and those authors suggest that shells also can provide accurate indication of metal pollution and a historical record of metal content throughout the organism's life history (Kupekar and Kulkarni, 2014; McClintock et al., 2014). Hence, it would be interesting to investigate the role of *A. crenata* shell as a storage matrix for toxic metals, therefore, an indicator of estuarine trace metal pollution.

Apart from the waterborne toxicity to Cd reported in this study, there are no metal toxicity studies conducted to investigate effect of dietary metal exposure in this organism. Understanding the mechanisms of metal accumulation from different pathways and toxic responses are essential for the development of biodynamic models which allow extrapolation between different species on the basis of shared pathways of impact.

The results of population attribute studies demonstrate that in future estuarine toxicity testing, effect of multiple stressors such as sediment trace metals, organic matter, nutrients, salinity and tidal level, should be included to obtain a more accurate interpretation of role of *A. crenata* population characteristics as bioindicators of estuarine health.

Apart from trace metal contamination, it is worth studying bioaccumulation of organic contaminates in the mud snail tissues as a number of studies have reported elevated levels of organic pollutants such as organochlorine pesticides (e.g. DDT), organophosphates (e.g. diazinon) and PAHs in NZ estuaries (Scobie et al., 1999; Ausseil, 2011; Cavanagh and Ward, 2014;). Furthermore, it would be worth studying multiple and possibly interacting contaminants including organic pollutants and trace metals.

In the present study, field collected snails have shown few correlations between snail physiological/biochemical responses and certain environmental variables. This could be attributed to the confounding effects or interaction between two or more environmental variables. Therefore, cage experiments using *A. crenata* would be helpful to overcome such environmental influences

and obtain more precise results. Cage experiments were successfully used to measure survival and growth of *A. crenata* in relation to the changing environmental nutrients (Marsden and Baharuddin, 2014). Such experiments might be useful to measure the effects of contaminants following remediation measures.

Apart from metal and nutrient contaminants, industrial and agricultural activities are also discharged a variety of organic pollutant into NZ estuaries including DDT, aldrin, dieldrin and PCBs (Hume et al., 1989; Cavanagh and Ward, 2014). The concentrations of these organic pollutants posed a potential health risk to humans even at low consumption rate (Stewart et al., 2011). In the present study induced GST activity in field collected snails suggested that this could be related to presence of organic contaminants in the environment. Further studies on accumulation of organic pollutants and biomarker responses in the mud snail, *A. crenata* could be helpful to understanding the cumulative effects of environmental contaminants.

7.4 CONCLUSIONS

In conclusion, while this thesis research has identified the specific effects of certain contaminants there are still many studies, including experimental laboratory studies which will advance the biomarker approach to environmental monitoring. The methods used in this thesis however can be used in other environment, for example using fresh water gastropods or bivalves to assess the effects of specific contaminants. Finally, this thesis research was undertaken on an endemic mud snail and, because species are likely to show species-specific responses, there is a need for more biomarker studies on New Zealand endemic organisms especially where these are classified as endangered or rare.

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